SIGNALLING AND MEDIATORS OF ANGIOPOIETIN-1 IN ENDOTHELIAL CELLS

by

Nelly Abdel Malak

Division of Experimental Medicine, Department of Medicine
Faculty of Medicine, McGill University
Montreal, Quebec, Canada

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DEDICATION

To my mom, Nahida

To my dad, Abbouda

To my sister and brother-in-law,
Rola and Bashar

To my sister, Rima

To my grandmother, Samira

To all my family,

I dedicate my doctoral thesis to you,
For your faith in me,
For your constant love and
continuous support.

ABSTRACT

Angiopoietin-1 (Ang-1), the main ligand for the endothelial cell (EC)-selective Tie-2 receptors, promotes survival, proliferation, migration and differentiation of these cells. Despite its importance in various aspects of vascular biology, the mechanisms of action of the Ang-1/Tie-2 receptor pathway have not been fully explored.

To identify the downstream modulators of Ang-1, we evaluated changes in the transcriptome of human umbilical vein endothelial cells (HUVECs) treated with Ang-1 protein for four hours by employing the oligonucleotide microarray technology. Eighty-six genes were significantly upregulated by this treatment and forty-nine genes were significantly downregulated. These genes are involved in the regulation of cell cycle, proliferation, apoptosis, transcription and differentiation. Furthermore, we found that the Erk1/2, PI3-Kinase and mTOR pathways are implicated in promoting gene expression in HUVECs in response to Ang-1. Analysis of the microarray data employing the Ingenuity Pathways analysis software to place the regulated genes in the context of biological networks revealed several highly connected nodes including the chemokine Interleukin-8 (IL-8) and the transcription factor Early growth response-1 (Egr-1). Due to the importance of these genes in promoting angiogenesis, we decided to evaluate their roles in Ang-1/Tie-2 receptor signaling and biological effects.

Ang-1 induced IL-8 expression in a time- and dose-dependent manner in ECs through both transcriptional and post-transcriptional mechanisms. To study the functional role of Ang-1-induced IL-8, we generated HUVECs that overexpress Ang-1. In these cells, neutralizing IL-8 significantly reduced EC proliferation and migration. IL-8 promoter activity experiments and gel shift assays revealed the involvement of the transcription factor AP-1 in Ang-1-induced IL-8. Ang-1 stimulated the phosphorylation of c-Jun through activation of Erk1/2, JNK and PI-3 kinase pathways. Similarly, Ang-1 provoked the expression and DNA binding of Egr-1 in HUVECs. Employing siRNA and DNAzyme to specifically knock-down Egr-1, we found that Ang-1-induced Egr-1 also promotes EC proliferation and migration.

We conclude that Ang-1 provokes a coordinated response intended to promote EC survival, proliferation, and angiogenesis and to inhibit EC apoptosis. Ang-1 induces EC

proliferation and migration in part through the secretion of the soluble mediator Interleukin-8 and through induction of the transcription factor Egr-1.

RÉSUMÉ

L'Angiopoiétine-1 (Ang-1), ligand principal du récepteur Tie-2 qui est sélectivement exprimé sur les cellules endothéliales (CEs), favorise la survie, la prolifération, la migration et la différentiation de ces cellules. Malgré son importance dans différents aspects de la biologie vasculaire, les mécanismes d'action de la voie Ang-1/Tie-2 n'ont pas été entièrement explorés.

Dans l'objectif d'identifier des médiateurs en aval de l'Ang-1, nous avons étudié par la technologie des puces à oligonucléotides « microarray » le transcriptome des cellules endothéliales de la veine ombilicale humaine (CEVOH) traitées pendant quatre heures avec de l'Ang-1. Quatre-vingt-six gènes ont été sur-exprimés et quarante-neuf sous-exprimés d'une manière significative par ce traitement. Ces gènes sont impliqués dans la régulation du cycle cellulaire, la prolifération, l'apoptose, la transcription et la différentiation. De plus, nous avons trouvé que les voies de signalisation de Erk1/2, PI-3 kinase et mTOR promeuvent l'expression des gènes des CEVOHs en réponse à l'Ang-1. Pour analyser les données des microarrays, nous avons utilisé le logiciel d'analyse « Ingenuity Pathways » pour situer les gènes régulés dans le contexte d'un réseau biologique. Ceci nous a permis d'identifier plusieurs nœuds fortement liés dont la chemokine Interleukine-8 (IL-8) et le facteur de transcription Early growth response-1 (Egr-1). En raison de l'importance de ces gènes dans la promotion de l'angiogenèse, nous avons décidé d'évaluer leurs rôles et leurs effets biologiques dans la voie de signalisation Ang-1/Tie-2.

Ang-1 a induit l'expression d'IL-8 dans les CEs d'une façon dose- et temps-dépendante par l'intermédiaire des mécanismes transcriptionel et post-transcriptionel. Pour étudier la fonction de l'IL-8 induite par l'Ang-1, nous avons produit des CEVOHs sur-exprimant ce ligand. La neutralisation de l'IL-8 dans ces cellules réduit d'une manière significative la prolifération et la migration cellulaires. L'étude de l'activité du promoteur de l'IL-8 ainsi que les essais de retard sur gel ont prouvé la participation du facteur de transcription AP-1 dans l'induction de l'IL-8 par l'Ang-1. Ang-1 stimule la phosphorylation de c-Jun à travers l'activation des voies de signalisation de Erk1/2, JNK et la PI-3 kinase. Également, Ang-1 provoque dans les CEVOHs l'expression de Egr-1 et sa liaison à l'ADN. En utilisant des siRNAs et des ADNzymes pour spécifiquement

rendre silencieux l'expression du gène de l'Egr-1, nous avons trouvé que l'induction d'Egr-1 par Ang-1 favorise aussi la prolifération et la migration des CEs.

Nous concluons qu'Ang-1 provoque une réponse coordonnée prévue pour favoriser la survie, la prolifération et l'angiogenèse ainsi que pour inhiber l'apoptose des CEs. Ang-1 induit partiellement la prolifération et la migration des CEs à travers la sécrétion du médiateur soluble IL-8 et l'induction du facteur de transcription Egr-1.

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LIST OF ABBREVIATIONS

ABIN-2	A20 binding inhibitor of NFκB activation-2
ALI	Acute lung injury
ANGII	Angiotensin II
AP-1	Activator protein-1
Ang	Angiopoietin
Angptl	Angiopoietin-like protein
ARE	Adenine and uridine rich elements
Asp	Aspartic acid
BBB	Blood brain barrier
BMK1	Big Mitogen-activated protein kinase1
bp	Base pair
Caspase	Cysteinyl Aspartate Specific Protease
CBP	CREB-binding protein
CEC	Circulating endothelial cell
CHX	Cycloheximide
Csk	C-terminal Src kinase
DBD	DNA-binding domain
Dok-R	Downstream-of-kinase-related
DSP	Dual specificity protein tyrosine phosphatase
E	Embryonic day
ENOS	Endothelial cell nitric oxide synthase
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EPC	Endothelial progenitor/precursor cell
ERK1/2	Extracellular signal-regulated kinase1/2

FGF	Fibroblast growth factor
FN	Fibrinogen
GAP	GTPase activating protein
GAPDH	Glyceraldehyde phosphate dehydrogenase
GEF	Guanine nucleotide exchange factors
GPCR	G-protein-coupled receptor
GSK3	Glycogen synthase kinase-3
HB-EGF	Heparin binding EGF-like growth factor
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor 1
HMEC	Human dermal microvascular endothelial cells
HNF-1	Hepatocyte nuclear factor-1
H_2O_2	Hydrogen peroxide
HSC	Hematopoeitic stem cell
HSP	Heat shock protein
HSPG	Heparan sulfate proteoglycan
HuR	Hu antigen receptor
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of apoptosis
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin-like growth factor
IL	Interleukin
IRF-1	IFN regulatory factor-1
KSRP	KH-type splicing regulatory protein
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
LMW-PTP	Low-molecular-weight protein tyrosine phosphatase
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemoattractant protein-1
MKP-1	MAPK phosphatase-1
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MMP	Matrix metalloproteinase
MTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
NF-κB	Nuclear Factor-kappa beta
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PAF	Platelet activating factor
PAK	p21-activated kinase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PH	Pleckstrin homology
PI	Phosphatidylinositol/phosphoinositide
PI3-Kinase	Phosphatidylinositol-3' kinase
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTP	Protein tyrosine phosphatase
PVDP	Polyvinylidene fluoride
Rho	Ras-homologous
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S6K	p70 S6 kinase
S1P	Sphingosine-1-phosphate
SAPK/JNK	c-Jun N-terminal kinases/stress-activated kinases
SDS	Sodium dodecyl sulfate

SE	Standard error
SEM	Standard error on the mean
SH	Src homology
SHP-2	Src homology domain 2 (SH2)-containing tyrosine phosphatase-2
SK-1	Sphingosine kinase-1
Smac	Second mitochondria-derived activator of caspase
STAT	Signal transducers and activators of transcription
TEK	Tunica interna endothelial cell kinase
TEY	Threonine-glutamic acid-tyrosine
TF	Tissue factor
TGF-β	Transforming growth factor-beta
TGY	Threonine-glycine-tyrosine
Tie	Tyrosine kinase with immunoglobulin and EGF-like domains
TNFα	Tumor necrosis factor alpha
TPY	Threonine-proline-tyrosine
TTP	Tristetraprolin
Tyr	Tyrosine
uPA	Urokinase plasminogen activator
UTR	Untranslated region
UV	Ultraviolet
VE-cadherin	Vascular endothelial-cadherin
VEGF/VPF	Vascular endothelial growth factor/vascular permeability factor
VE-PTP	Vascular endothelial protein tyrosine phosphatase
WM	Wortmannin
WPB	Weibel Palade bodies
WT1	Wilm's tumor suppressor

CONTRIBUTION OF AUTHORS

For all manuscripts, Dr Sabah N. Hussain was instrumental in guiding my research. He provided insightful input upon which I based my research. He guided me in interpreting the data and in structuring the manuscripts. I performed most or all experiments for all manuscripts, generated hypotheses and also optimized and designed most of the protocols.

Manuscript 1: Transcriptome of Angiopoietin-1 activated human umbilical vein endothelial cells.
Endothelium, 14(6): 285-302, 2007

Although I performed all experiments for this manuscript, Rania Harfouche provided technical assistance for the Real-Time PCR experiments for Egr-1 mRNA expression.

Manuscript 2: Angiopoietin-1 induces endothelial cell proliferation and migration through AP-1 dependent autocrine production of interleukin-8.
Blood, 111(8): 4145-54, 2008

Even though I performed all experiments for this manuscript, I could not have generated this manuscript without the fruitful collaboration with the co-authors:

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Manuscript 3: Early growth response-1 mediates Angiopoietin-1-induced endothelial cell proliferation and migration.

To be resubmitted to Arteriosclerosis, Thrombosis and Vascular Biology, 2008

Although I performed all experiments for this manuscript, again, with valuable input and guidance from Dr Hussain, the co-authors were instrumental in generating this manuscript:

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- Dominique Mayaki provided instrumental help in generating the real-time PCR data.

CHAPTER 1

CHAPTER 1: LITERATURE REVIEW

1.1. The endothelium and the vascular system

1.1.1. Introduction

Blood vessels ensure the proper delivery of nutrients, oxygen, blood and immune cells to various tissues. They are structurally and functionally highly heterogeneous but are essentially composed of an endothelial cell (EC) monolayer, known as the endothelium, which is surrounded by organ-specific mural cells and embedded in an extracellular matrix (1;2). The endothelium serves several important functions, including the separation of blood and tissues, maintenance of blood fluidity and solute diffusion, regulation of leukocyte adhesion and migration, prevention of thrombosis, regulation of organ blood flow, coagulation, vasodilation, wound healing and angiogenesis (1;2).

Angiogenesis, the formation of new blood vessels from pre-existing ones, is required for proper homeostasis during embryonic development, as well as in adults. Rates of angiogenesis are determined by the balance between various promoters and inhibitors of the process. Excessive angiogenesis is pathogenetically-associated with many abnormal conditions, including tumour growth, arthritis, diabetic retinopathy and other inflammatory diseases, where new vessels nourish diseased tissue and destroy healthy tissue (3-6). Insufficient angiogenesis, in contrast, can be detrimental to the proper functioning of the myocardium during recovery from ischemic heart diseases (6-9). Hence, understanding the molecular mechanisms that govern the process of angiogenesis is of prime importance in the quest for identifying new therapeutic targets for angiogenesis-related diseases.

Genetic studies in mice have shed light onto the molecular mechanisms involved in the process of angiogenesis. A growing body of evidence implicates the endothelial cell-specific receptor tyrosine kinases (RTKs), namely Flt-1 type VEGF receptor (Flt-1) and kinase insert domain receptor (KDR or Flk-1), which are receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), and the tyrosine kinase receptors Tie-1 and Tie-2 (10). Disruption of Flt-1 and KDR/Flk-1 genes leads to embryonic lethality between days 8.5 and 9.5 (11;12), whereas disruption of Tie-1 or Tie-

2 receptors leads to lethality at a later date (13), suggesting that VEGF receptor ligands appear at a later stage than do Tie-1 and Tie-2 ligands. Thus far, four Tie-2 ligands (angiopoietin-1, -2, -3 and -4) have been characterized, whereas the Tie-1 receptor is still considered an orphan receptor. Angiopoietin-1 (Ang-1), the most-studied Tie-2 agonist, promotes angiogenesis, is involved in vessel remodelling and possesses vascular protective effects, including inhibition of plasma leakage and vascular inflammation, promotion of EC survival and overall integrity of the endothelium.

Despite major recent advances in understanding the roles of Ang-1 in vascular physiology and pathophysiology, little is yet known about its signalling pathways and mechanisms of action on EC proliferation and migration, two important steps in the process of angiogenesis. The general objective of this thesis, therefore, is to identify mechanisms of action of angiopoietin-1 that act through the receptor Tie-2 in mediating EC function.

1.1.2. Blood vessel formation: vasculogenesis and angiogenesis

During vertebrate embryonic development, the cardiovascular system is the first organ system to develop (14). The process of blood vessel formation involves two distinct mechanisms: vasculogenesis and angiogenesis. Vessels initially originate from the mesoderm where hemangioblasts, the precursors of both angioblasts and hematopoietic cells, can be found. These precursor cells then differentiate into clusters of epithelioid cells called blood islands that give rise to both endothelial and hematopoietic cells (15). ECs from adjacent blood islands coalesce and form a vascular plexus made up of primitive endothelial tubes (16). The term vasculogenesis is used to describe this early step of hemangioblast cell determination and differentiation and their organization into a primitive vascular network (17). Although vasculogenesis occurs mainly during embryonic development, it has been recently suggested that vasculogenesis might also occur in the adult in pathological conditions such as tumour growth, tissue ischemia and vascular grafts, where haematopoietic stem cells (HSCs) and bone marrow-derived endothelial progenitor cells (EPCs) are recruited to sites of neovascularization (18-22).

The formation of new blood vessels by remodelling and expansion of the primitive vascular network occurs through the process termed angiogenesis (17).

Angiogenesis occurs in four different ways: 1) sprouting angiogenesis, or the formation of new sprouting capillaries from pre-existing vessels; 2) non-sprouting angiogenesis or intussusception, which is the splitting of pre-existing vessels by the formation of transcapillary meshes containing collagen fibrils, a process demonstrated initially in the lungs; 3) anastomosis, or the tip-to-tip or tip-to-sprout fusions of adjacent blood vessels; and 4) intercalated growth, whereby ECs proliferate without sprouting leading to the enlargement of neovessels (23-27).

Sprouting angiogenesis, the most extensively studied mechanism of angiogenesis, takes place in four successive steps (28;29). In the initial step, the parent vessel dilates, leading to spreading and elongation of ECs (ECs) and increased EC responsiveness to angiogenic stimuli (30). This is followed by increased vascular permeability, which allows plasma proteins to infiltrate into the surrounding tissue and form a supporting framework for subsequent EC migration (28). Activated ECs secrete several endothelial proteases of the plasminogen activator and matrix metalloproteinase (MMP) families. These proteases degrade matrix molecules, facilitating subsequent EC invasion of the vessel basal lamina and leading to the release of growth factors sequestered in the extracellular matrix (ECM) (31).

In the second and third steps, ECs proliferate and migrate through the degraded matrix. Molecules of the integrin family that are involved in cell-ECM interactions mediate EC migration (28;29). The $\alpha_v\beta_3$ integrin is a particularly important player in angiogenesis. Its expression is induced following stimulation by angiogenic factors (32). In addition to binding several ECM proteins, $\alpha_v\beta_3$ integrin binds directly to proteolytically active MMP-2, resulting in localized matrix degradation at the invasive/migratory site of vascular cells, allowing further vessel invasion (33).

In the last step of sprouting angiogenesis, ECs differentiate into tube-like structures, adhere to the basement membrane that they have secreted and subsequently form a lumen. Laminin and collagen I are the major basement membrane components that induce this differentiation as they promote cell adhesion by binding to integrins (28;34). The recruitment of pericytes and smooth muscle cells (SMCs) to sites around the basement membrane further stabilizes these vessels. These mural support cells secrete

additional ECM components and inhibit further EC proliferation, maintaining the mature and quiescent state of the neovessels (2).

Whereas vasculogenesis occurs mainly during embryonic development, angiogenesis is primarily involved in blood vessel growth in the adult, both under physiological conditions, as in the female reproductive system, and pathological conditions, as in malignant, ocular and inflammatory disorders (3;6;35). Under both conditions, angiogenesis is initiated when the ratio of pro-angiogenic factors, such as VEGF and fibroblast growth factor (FGF), to anti-angiogenic factors, such as interferon and angiostatin, is tilted towards the former (36;37). This phenomenon is called the "angiogenic switch". Numerous mediators have been implicated in various aspects of angiogenesis. **Of particular interest for this thesis** are the roles of the growth factor Angiopoietin-1 and its receptor Tie-2, the immediate early gene early growth response-1 (Egr-1) and the chemokine interleukin-8 (CXCL8).

1.2. Molecular mechanisms of vasculogenesis and angiogenesis

1.2.1. Growth factors and growth factor receptors involved in vasculogenesis and angiogenesis

Early studies in the avian embryo revealed that basic fibroblast growth factor (bFGF, FGF-2) is the main mediator responsible for both the induction of the mesoderm from pre-mesodermal precursors and the subsequent formation of primitive capillary-like vascular networks (38). bFGF initiates vasculogenesis by inducing the expression of the earliest *in vivo* marker for the hemangioblastic cell lineage, the vascular endothelial growth factor receptor-2 (VEGF-R2, KDR/Flk-1), on hemangioblast cells (39;40). The requirement for Flk-1 in vasculogenesis has been demonstrated in mice deficient in Flk-1, as these mice fail to form a primitive vascular plexus (12).

The expression of VEGF receptors constitutes the link between vasculogenesis and angiogenesis (40). VEGF, one of the most potent pro-angiogenic cytokines (41), was first discovered in 1989 as an EC specific growth factor (42;43). *In vivo*, its expression correlates with physiological and pathological vascularisation in the embryo and the adult, respectively (44;45). The VEGF family consists of six members: VEGF-A, -B, -C,

-D, -E and placenta growth factor (PlGF) (46;47). VEGF-A (also known simply as VEGF) is the most biologically active and most abundant member (47). It promotes vasculogenesis, angiogenesis, lymphangiogenesis and haematopoiesis by inducing EC proliferation, migration and survival, as well as vascular leakage (41;48;49). The specificity of VEGF for ECs depends on the restricted expression of its tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1, Flt-1) and VEGF receptor-2 (VEGFR-2, KDR/Flk-1), by the endothelium (50-53). Most of the biological and pathological actions of VEGF are mediated through the VEGFR-2 (41). However, both VEGFR-1 and -2 play important roles in mediating the effects of VEGF-A. During vasculogenesis, VEGFR-2 is required for angioblast differentiation (12), whereas VEGFR-1 is required later on for proper assembly of angioblasts into functional blood vessels (11). In addition, VEGFR-2 promotes robust EC proliferation, whereas both VEGFR-1 and -2 are required for EC migration (54). VEGFR-3 is the third member of the VEGFR family. It is expressed on lymphatic ECs and mediates the effects of VEGF-C and VEGF-D on embryonic vasculogenesis and lymphogenesis (55;56). Although VEGF receptors are absolutely required throughout the process of vasculogenesis and angiogenesis, Tie receptors have also recently been identified as important modulators of the angiogenic response (23;57).

1.3. Tie receptors as modulators of angiogenesis

Tie (**t**yrosine kinase with **i**mmunoglobulin and **e**pidermal growth factor homology domains) constitutes a relatively new class of RTKs that are preferentially expressed by vascular ECs and their precursors. This class is comprised of two members, namely, Tie-1 (also known as Tie) and Tie-2 (also known as Tek) (58-63).

1.3.1. Structure and expression profile of Tie receptors

Tie-1 and Tie-2 share a similar overall structure, with the intracellular regions being more conserved than the extracellular regions (63) (Figure 1.1). Both possess a unique multiple extracellular domain composed of two immunoglobulin (Ig)-like loops, separated by a cluster of three epidermal growth factor (EGF)-like repeats, and followed by three fibronectin (FN) type III repeats (60;61;63). The first 360 amino acids

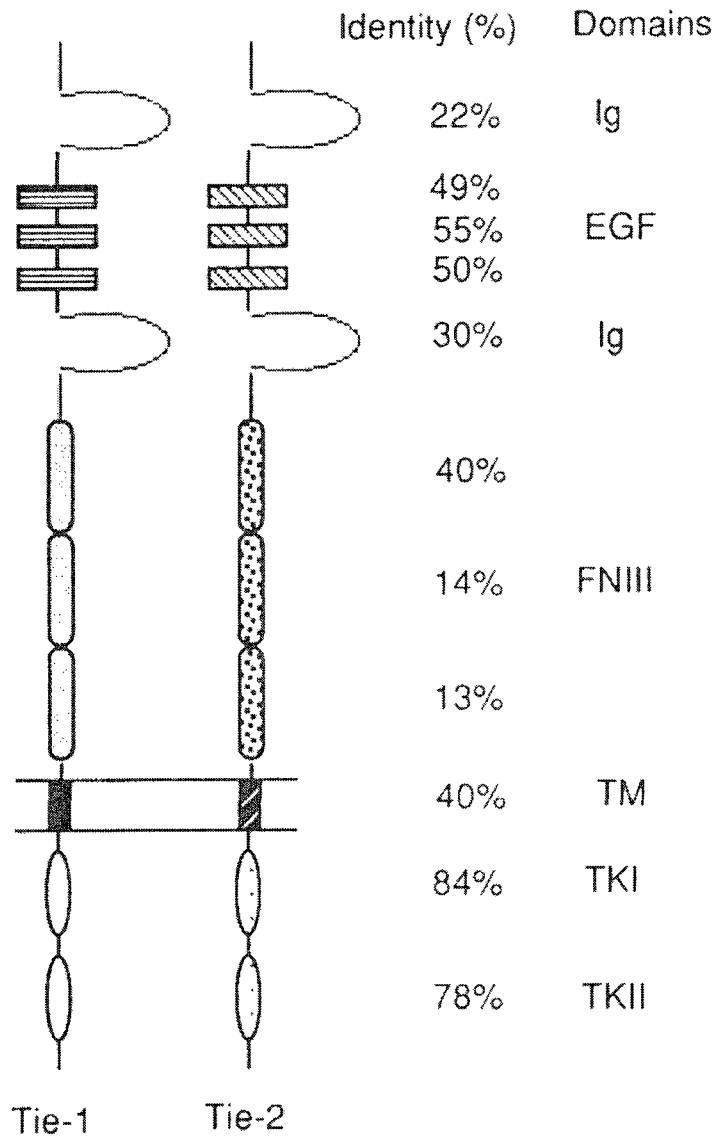

Figure 1.1: Domain structure of Tie-1 and Tie-2 receptors.

% Amino acid identities between mouse Tie-1 and Tie-2 receptors within each domain are indicated at the right. Ig: immunoglobulin; TM: transmembrane; TK: tyrosine kinase. Sato *et al.* Proc.Natl.Acad.Sci.USA 90: 9355-9358, 1993

encompassing the first IgG loop and the three EGF-like repeats are responsible for ligand binding (64). The intracellular cytoplasmic regions contain two tyrosine kinase domains with a 21 amino acid kinase insert region devoid of any tyrosine residue (58;63). These tyrosine kinase domains are highly conserved between Tie-1 and Tie-2, as they share 80% identity at the amino acid sequence level (63). The functions of Tie-2 have been well described in ECs, however the roles of Tie-1 are less clear, mainly due to the lack of an identified ligand. Recent studies suggest that Tie-1 exists in a pre-formed complex with Tie-2 in quiescent ECs, an association mediated by its kinase domain (65).

During embryonic development, Tie-1 and Tie-2 are expressed by ECs of the endocardium and the vascular endothelium (58;59;62;63;66;67). Whereas Tie-2 transcripts are first detected in ECs at embryonic day 7.5 (E7.5), Tie-1 expression begins slightly later, at E8.0. This expression continues throughout development (66;67). The transcriptional activation of both Tie receptors depends on the presence of a consensus octamer element in their promoters, which interacts with a protein complex comprised of the ubiquitous transcription factor Oct-1 and an as yet unidentified endothelial-cell-specific cofactor (68;69). In the adult vasculature of rats and mice, both Tie-1 and Tie-2 expression is detected in ECs in a wide range of adult tissues, although they seem to be most abundant in the lung (59;63;70;71). In the adult, however, the expression of both genes is less pronounced than in embryos (63).

A growing body of evidence suggests the presence of a subpopulation of Tie-2 receptors in non-ECs. For instance, Tie-2 expression in aortic mural precursor cells mediates Ang-1- and Ang-2-induced recruitment of these cells to the developing microvessels in the rat aorta model of angiogenesis (72). In addition, Tie-2 receptors have been recently detected on human neutrophils, where they promote inflammatory responses, both by inducing the migration of these cells toward Ang-1 and Ang-2 and by enhancing their adhesion to ECs in response to both ligands (73;74). Furthermore, Tie-2 receptors have been found in thyroid epithelial cells, where they contribute to goitrogenesis, and in cells of the peripheral and autonomic nervous system, where they play a role in neuroprotection and neuritogenesis (75-78). Finally, Tie-2 expression has been localized on EPCs and HSCs derived from bone marrow, peripheral blood and umbilical cord blood cells, where they promote re-endothelialization and tumour

angiogenesis, as well as stem cell quiescence and interaction with microenvironments (79-84). An explanation for this latter finding might be the hypothetical existence of hemangioblasts, the common precursor of both hematopoietic and endothelial progenitor cells, although the presence of Tie-2 in other cell types requires further investigation (23;84;85).

1.3.2. Biological roles of Tie receptors during embryonic development

Analysis of Tie-1 and Tie-2 null mutant mice has provided much of the information regarding their respective roles in embryonic development. Transgenic mice expressing a dominant-negative form of Tie-2, or mice with a disrupted Tie-2 gene (Tie-$2^{-/-}$), both die between E9.5 and E10.5 due to severe haemorrhage, EC loss and detachment of the endocardium from the underlying myocardium (13;86). A possible role of Tie-2 in EC survival and vascular maintenance has also been observed in Tie-2 transgenic mice (87). Tie-1 plays an important role in vessel formation, but at a late stage. The Tie-1 null mutant phenotype results in lethality between E13.5 and immediately after birth, due to loss of vessel integrity, haemorrhage and edema, especially in the lungs (13;88). Although Tie-2 is required earlier than is Tie-1 during embryonic angiogenesis, both receptors are necessary during the late phases of organogenesis, as well as in the adult vasculature. Simultaneous genetic deletion of both Tie-1 and Tie-2 results in a more severe phenotype than that which results from a single knockout (89). This cooperation between receptor family members having distinct functions suggests a complex role of the Tie receptors in the vascular system.

1.4. Immediate early genes involved in angiogenesis

Immediate early genes are those that are induced rapidly following cellular stimulation by a diverse variety of ligands and function to link cellular activation with cellular responses reflected by changes in the expression of target genes. Of particular importance to this thesis is the involvement in the angiogenic process of transcription factor activator protein-1 (AP-1) and early growth response-1 (Egr-1).

1.4.1. Transcription factor AP-1

Transcription factor AP-1 is a dimer consisting of members of the Jun and Fos families of proteins (90). The Fos proteins, namely c-Fos, FosB, Fra-1 and Fra-2, are not able to form homodimers but they can heterodimerize with members of the Jun family (c-Jun, JunB and JunD). The latter family members can homodimerize and heterodimerize with other Jun or Fos proteins to form transcriptionally active complexes (90;91). In addition to Fos proteins, Jun proteins are also able to heterodimerize with members of the activator transcription factor (ATF) family (92). c-Jun constitutes the major component of the AP-1 transcription factor complex and is the most potent transcriptional activator in its group (93;94).

AP-1 is activated following stimulation with growth factors, cytokines, hormones, cell-matrix interactions, bacterial and viral infections and physical and chemical stresses. These stimuli are able to activate mitogen-activated protein kinases (MAPKs), which in turn induce the activity of AP-1 (95). More specifically, serum and growth factors activate the extracellular regulated kinase (ERK) subclass of MAPKs, which translocate to the nucleus and then phosphorylate and enhance the transactivation potential of the ternary complex factors (TCFs). TCFs then bind to the c-Fos promoter and induce its transcription (96). In addition, Fra-1 and Fra-2 are directly phosphorylated by activated ERK, possibly increasing their DNA binding in combination with c-Jun (97). Pro-inflammatory cytokine induced AP-1 activity is mainly mediated by the p38 and stress-activated protein kinase (SAPK)/C-Jun N-terminal kinase (JNK) MAPK pathways (95). p38 directly phosphorylates and activates ATF2 and TCFs, which leads to upregulation of c-Jun expression (98). Activated JNK translocates to the nucleus and phosphorylates two serine residues (Ser^{63} and Ser^{73}) in the transactivation domain of c-Jun, thereby increasing its transcriptional activity (99;100). In addition, c-Jun activity is also enhanced by de-phosphorylation of specific serine and threonine residues in its DNA binding domain (101). Hence, the activation of c-Jun is regulated at several levels, including the transcriptional and post-transcriptional levels, and at the level of its interaction with other transcription factors, which either enhance or repress its activity (102).

The biological function of AP-1 is mediated by its direct effect on gene expression. Specifically, c-Jun promotes cellular proliferation by increasing the

expression of pro-proliferative genes such as cyclin D1 and inhibiting anti-proliferative genes such as p21 and p53 (103;104). In addition, AP-1 regulates the expression of several chemokines, including CXCL8 and growth-regulated oncogene-alpha (GRO-α) (105-108).

1.4.2. Early growth response-1

Early growth response-1 (Egr-1) is a zinc finger transcription factor first identified by Sukhatme *et al.* in 1988 (109). Alternatively, it is known as Zif268, nerve growth factor induced-A (NGFI-A), Krox24 or TIS8 (110-113). Egr-1 is an immediate-early response gene because its expression is dramatically and rapidly induced in many cell types and in response to a variety of stimuli, including growth factors, cytokines, hypoxia, shear stress and injury (114-116). Egr-1 regulates the expression of a wide array of genes, thereby coupling the extracellular stimuli to long-lasting responses (117). The Egr family of transcription factors also consists of several other members, including Egr-2 (Krox20), Egr-3 and Egr-4 (NGFI-C) that share 81-93% similarity with Egr-1 at the level of the DNA binding domain, which is composed of three zinc-finger motifs. Egr transcription factors are involved in several processes in a variety of cell types, including cell growth, differentiation and apoptosis in response to extracellular stimuli (115;118;119).

Further insights into the roles of Egr family members have been gained from genetic experiments in mice. Genetic deletion of Egr-1 results in female infertility as a result of a significant reduction in the levels of luteinizing hormone β (120). $Egr2^{-/-}$ mice die shortly after birth (within 2 weeks) due to defects in hindbrain development and peripheral nerve myelination (121;122). Disruption of the Egr-3 gene leads to loss of muscle spindles, resting tremors, sensory ataxia and an augmented frequency of perinatal death (123;124). Finally, Egr-4-deficient mice appear phenotypically normal although males are infertile due to germ cell apoptosis and defects in spermiogenesis (125).

Expressions of Egr-2 and -3 are induced in fibroblasts, lymphocytes and monocytes in response to mitogenic stimuli, whereas much less is known regarding the expression of Egr-4 (118;126;127). Both Egr-2 and Egr-3 negatively regulate T cell activation whereas Egr-2 is involved in monocyte activation and differentiation

(126;128;129). In addition, Egr-2 is associated with cognitive functions since its expression is enhanced in the forebrain of mice subjected to different odour and texture stimuli (130). Of particular note is that VEGF significantly induces the expression of all four Egr family members in human umbilical vein ECs (HUVECs) (131).

Recent work has revealed that Egr-3 is the primary Egr family member that is induced downstream from the VEGF-A/VEGFR-2/PKC pathway in ECs and that it plays a role in EC proliferation, migration and differentiation into tube-like structures, thus promoting angiogenesis (132). Moreover, both Egr-1 and Egr-3 are implicated in thymocyte survival, proliferation and differentiation, thus exhibiting functional redundancy, although they possess distinct roles in learning and memory (133-135). As for Egr-4, one study has shown that it acts as a transcriptional repressor of its own promoter as well as that of c-Fos (136), whereas other reports have demonstrated the importance of Egr-4 in the induction of both TNFα and interleukin-2 (IL-2) in antigen-stimulated T lymphocytes and also of the neuron-specific K^+/Cl^- cotransporter (KCC2) (137;138). Although Egr-1 and Egr-4 have also been shown to have redundant functions in terms of luteinizing hormone regulation in male mice, they possess distinct and specific DNA binding sites on their target genes (136;139). Egr-4 can form heterodimeric complexes with either of two other transcription factors of the nuclear factor of activated T cells (NFAT) family, a property shared with Egr-1, and the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1(NFκB) factors p50 and p65, a property shared with Egr-3, thereby indicating a crucial role of these factors in regulating the expression pro-inflammatory genes such as IL-2, TNFα and intercellular adhesion molecule-1 (ICAM-1) (137;140).

1.4.2.1. Structure and regulation of Egr-1 expression

The Egr-1 gene consists of two exons and one intron and is located on chromosome 5 (109). The protein has a modular structure and is composed of three domains - an amino-terminal activation domain, a repression domain and a carboxy-terminal DNA binding domain (141) (Figure 1.2). The extended transcriptional activation domain is located between amino acids 1 and 281 and directly interacts with the transcriptional co-activators cAMP-response-element-binding-protein (CBP) and p300,

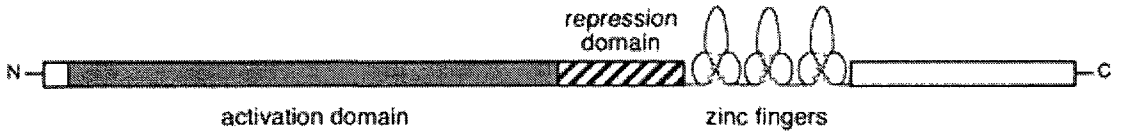

Figure 1.2: Modular structure of the transcription factor Egr-1.

Egr-1 protein is composed of three domains: an amino-terminal activation domain, a repression domain (role as a binding site for the co-repressor proteins Nab1 and Nab2) and a carboxy-terminal DNA binding domain.
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resulting in enhanced Egr-1-transactivation potential (142;143). The DNA binding domain is well characterized. It is situated between amino acids 332 and 416 and is composed of three zinc finger motifs through which Egr-1 specifically binds to the GC-rich DNA sequence 5'-GCG(T/G)GGGCG-3' (144;145). The inhibitory domain, located between the activation and DNA binding domains (amino acids 281 to 314), serves as a binding site for two corepressor molecules of Egr-1, namely nerve growth factor-induced (NGFI)-A-binding proteins-1 and -2 (NAB1 and NAB2) (143;146;147). Both NAB1 and NAB2 bind directly to Egr-1 and repress its biological activity (146-148). Whereas NAB1 is ubiquitously expressed at low levels in many cell types, NAB2 expression is induced by the same factors that stimulate Egr-1 expression and by Egr-1 itself, thus providing a negative feedback mechanism to regulate Egr-1 activity and prevent continuous transactivation of its target genes (147-151).

Egr-1 expression is rapidly induced in response to extracellular stimuli, including growth factors and cytokines, environmental stresses such as hypoxia, fluid shear stress and vascular injury (152). Human Egr-1 promoter harbours five serum response elements (SRE) that drive its transcription in response to extracellular stimulation (153). Activation of transcription by SRE requires the interaction between two transcription factors, serum response factor (SRF) and ternary complex factors (TCF) (154;155). Activation of the ternary complex factor Elk-1, downstream from ERK1/2 MAPK, has been linked to transactivation of Egr-1 (156-160). Similarly, activation of the ERK1/2 pathway by employing constitutively active MAPKK-1 (the kinase upstream from ERK1/2), or following stimulation with serum, platelet-derived growth factor (PDGF), FGF-2, hepatocyte growth factor (HGF), angiotensin II, lysophosphatidylcholine, or phorbol myristate acetate (PMA) strongly stimulates Egr-1 expression (161-166). Elk-1 is also activated downstream from the p38 and JNK MAPK pathways, leading to Egr-1 induction (167-170).

In addition to SRE, the Egr-1 promoter contains transcription factor SP-1 and AP-1 sites as well as cAMP response element (CRE) loci. The latter regulate Egr-1 transactivation through activation of the p38 MAPK pathway independently of cAMP (171-173). Intriguingly, Egr-1 protein inhibits its own gene expression by binding to its

own promoter at the EBS site, providing another negative feedback mechanism that controls Egr-1 expression (145).

It has been suggested that an association between ERK1/2 and Grb2-associated binder-1 (Gab1) through Gab1-growth factor receptor c-Met (Gab1-c-Met) binding leads to nuclear accumulation of Egr-1 (174). In addition to the aforementioned growth factors, VEGF, EGF and a combination of HGF and VEGF also induces Egr-1 expression in ECs (131;175;176). Aside from our recent study, which identified Egr-1 as a transcription factor that is highly induced by Ang-1 in ECs, no other research has, thus far, linked Egr-1 to Ang-1/Tie-2 signalling (177).

1.4.2.2. Regulation of gene expression by Egr-1

In addition to the regulation of its own promoter, binding sites for Egr-1 have been localized in the promoter regions of several other genes, such as those of the growth factors PDGF-A and PDGF-B (114;178;179), FGF-2 (180) and transforming growth factor β1 (TGFβ1) (181), ICAM-1 (182), TNFα (183), macrophage-colony-stimulating factor (M-CSF) (184), tissue factor (TF) (185), urokinase-type plasminogen activator (u-PA) (186) and membrane-type 1-matrix metalloproteinase (MT1-MMP) (187). VEGF and Flt-1 type VEGF receptor (Flt-1) have also been identified as Egr-1 target genes (188;189). Several of these gene products stimulate, in turn, the expression of Egr-1, thus creating positive feedback loops that serve to further amplify and sustain gene transcription through Egr-1, as is the case with PDGF-A and FGF (179;190;191). In most cases, Egr-1 cooperates with other transcription or regulatory factors to modulate gene expression. For instance, a synergistic interaction between Egr-1 and NFAT activates the transcription of both human IL-2 and TNFα in lymphocytes (137;192). Similarly, Egr-1 and steroidogenic factor-1 (SF-1) are both required to induce the expression of luteinizing hormone β (120;193).

In addition, Egr-1 cooperates with both AP-2 and glucocorticoid receptor (NR3C1) to activate the transcription of phenylethanolamine N-methyltransferase (PNMT) (194). Interestingly, Egr-1 associates with and sequesters the NFκB subunit Rel A (p65), preventing it from binding to its target promoters and leading to an inhibition of its transcriptional activity (195). In contrast, it has been shown that both Egr-1 and Rel A

synergistically cooperate to induce the expression of NFκB1 (p105) in T cells activated with PMA (196).

Perhaps the most interesting interaction occurs between Egr-1 and the transcription factors SP-1 and Wilms' tumour suppressor (WT1), all three of which share similar consensus binding sites that might overlap in many promoters (197;198). SP-1 is a ubiquitous zinc finger transcription factor that is found in almost all cell types. Generally, its expression increases in cells undergoing differentiation, reaching high levels and therefore could be required for the induction of tissue-specific genes (199). WT1 is a tumour suppressor gene that is expressed mainly in the kidney, where it plays a critical role in renal organogenesis and, when inactivated, in nephroblastoma development (Wilms' tumour) (200;201). The displacement of SP-1 by Egr-1 has been well studied in the vascular system, especially in the context of PDGF-A and PDGF-B promoters. SP-1 transactivation potential is weak when compared to that of Egr-1, and, in quiescent ECs, SP-1 binds to Egr-1/SP-1 DNA binding element and is responsible for basal levels of PDGF-A and -B transcription. Upon induction of Egr-1 expression following injury or growth factor stimulation, Egr-1 displaces SP-1 from the PDGF-A and -B promoters, resulting in enhanced transcription (114;178;202). Modulation of gene expression regulating this interplay between Egr-1 and SP-1 has been described for other genes as well, including HGF receptor, manganese superoxide dismutase (MnSOD), rearranged during transfection (RET) protooncogene, TF and sodium-calcium exchanger-1 (SLC8A1)(203-207). It is very possible, then, that Egr-1 does not modulate gene expression on its own, rather it works in concert with other factors to do so.

1.4.2.3. Biological roles of Egr-1 in the vascular system

In the vascular system, Egr-1 is expressed in ECs and smooth muscle cells (SMCs) and is upregulated following injury, hypoxia, shear stress and growth factor stimulation (114;178;208). In turn, Egr-1 upregulates the expression of a wide array of genes in ECs, including growth factors, cytokines, transcription factors, cell-cycle regulators and matrix components (209). Based on these findings, it has been proposed that Egr-1 might play a role in promoting angiogenesis and wound healing. Indeed, Egr-1 deficient mice are unable to mount an angiogenic response to subcutaneous matrigel

implants (152). In addition, agents that lower Egr-1 mRNA expression significantly reduce EC proliferation, migration and differentiation *in vitro*, inhibit VEGF-induced neovascularization in rat corneas and suppress tumour growth and tumour angiogenesis in mice (152). Similarly, the Egr-1 repressor NAB2 inhibits *in vitro* angiogenesis, Egr-1-driven growth factor production in ECs and vascular SMCs as well as matrigel plug neovascularization in mice (210;211). Mechanisms operating behind the pro-angiogenic properties of Egr-1 are not fully elucidated, however, such effects might be explained by the modulation of downstream gene targets, especially those of growth factors and their receptors. In fact, Egr-1 regulates, in the context of angiogenesis, the expression of several genes, including FGF-2, Flt-1, uPA, PDGF-A and -B, HGF and TGFβ1 (152;189;210;211). In addition, the expression of cysteine-rich, angiogenic inducer (CCN1), an angiogenic factor produced by SMCs that is involved in cell adhesion, proliferation and differentiation, is regulated by Egr-1 (212). Moreover, as demonstrated in a rodent model of dermal wounding, Egr-1 induces wound repair and enhances revascularization following vascular occlusion by promoting angiogenesis and enhancing collagen production (213;214).

In addition to physiological angiogenesis, Egr-1 is involved in pathologic angiogenesis, as in conditions such as atherosclerosis and restenosis. In fact, high levels of Egr-1 have been found in tissues and cells derived from human atherosclerotic lesions, specifically, SMCs and ECs (215;216). In murine experiments, Egr-1 knockout results in a significant reduction in atherosclerotic lesion size (217). This can be partly explained by the fact that inflammation is a crucial step in the initiation of early-stage atherosclerotic lesions (218). Several pro-inflammatory cytokines involved in the initiation and progression of atherosclerosis are known as Egr-1 target genes, including TNFα, monocyte chemoattractant protein-1 (MCP-1) and ICAM-1 (182;219-221).

In restenosis, or the renarrowing of blood vessels following successful unblocking, studies have demonstrated that vascular SMC migration and proliferation are crucial steps involved in the reconstriction of blood vessels following angioplasty (222). In addition, Egr-1, which is strongly and rapidly induced in vascular SMCs and ECs after arterial injury, is involved in the pathogenesis of restenosis by promoting the expression of several molecules, namely PDGF and TGFβ, that promote vascular SMC proliferation

and migration (114;117;197;223;224). Moreover, inhibition of Egr-1 expression has been shown to inhibit vascular SMC proliferation after *in vitro* mechanical injury and neointima formation in a rat carotid angioplasty model, and following stenting of pig coronary arteries *in vivo* (225-227).

1.5. Chemokines as mediators of angiogenesis

Chemokines, or chemotactic cytokines, are a large superfamily of structurally related small peptides (8-17 kDa) that were initially described as potent activators and chemoattractants for leukocyte subpopulations at sites of inflammation and injury (228;229). This superfamily can be subdivided into four major groups (CXC, CC, CX_3C, C) based on the positioning of the first two conserved cysteine residues in a four-cysteine motif at the N-terminus, which is either adjacent to or separated by one or more amino acids (230). Effects of chemokines are mediated through members of the G-protein coupled receptor family, termed CXCRs, CCRs, CX_3CRs and CRs. This unified nomenclature system was agreed upon and published following the XXII International Union of Pharmacology, since prior to that time chemokine ligands and their receptors were recognized under several names, making communication in the field a bit difficult (231).

Interestingly, chemokines and their receptors are promiscuous in that some ligands can bind several receptors and some receptors can bind several ligands, adding more complexity to their roles. These receptors are expressed on a variety of cell types including leukocytes, ECs and vascular SMCs indicating a role for chemokines beyond leukocyte physiology (231-234). In particular, the CXC family plays an important role in angiogenesis. To date, this family consists of seventeen ligands (CXCL1-17), each of which can bind to one or more of seven receptors (CXCR1-7) (235). CXC chemokines can be further subdivided into two groups, depending on the presence or absence of a three amino acid motif comprised of, glutamic acid-leucine-arginine (the ELR-motif), which appears immediately before the CXC motif. In general, the ELR-containing (ELR^+) CXC chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 promote angiogenesis, whereas the non-ELR ligands, such as CXCL4,

CXCL9, CXCL10, CXCL11, CXCL13 and CXCL14 inhibit angiogenesis (236-241). However, some exceptions to the ELR rule exist. In fact, both CXCL12 and CXCL16 lack the ELR motif and yet function as angiogenic factors (Figure 1.3 A) (242-246). To date, there is no human homologue for CXCL15, which, in the mouse, has been suggested as a factor in lung development and regulation of hematopoietic differentiation (247;248). Moreover, a function for CXCL17 has yet to be determined (235). To add another level of complexity to the chemokine system, it has been demonstrated that activation of specific receptors on ECs, rather than the absence or presence of a specific chemokine, dictates the angiogenic outcome. Based on this, both CXCR2 and CXCR4 are considered pro-angiogenic whereas CXCR3 is considered an inhibitor of angiogenesis (249-251). In terms of ligand specificity, CXCR1 recognizes only CXCL6 and CXCL8 whereas CXCR2 ligates all ELR^+ CXC chemokines. CXCR3 binds CXCL4, CXCL9, CXCL10 and CXCL11, whereas CXCR4 only binds CXCL12. CXCR5 and CXCR6 only relay the effects of CXCL13 and CXCL16, respectively. Recently, CXCR7 has been shown to mediate the effects of both CXCL11 and CXCL12 (Figure 1.3 B). Finally, the receptors for both CXCL14 and CXCL17 are yet to be determined (231;235;252).

1.5.1. Interleukin-8 (CXCL8):

Interleukin-8 is a prototypic ELR^+ CXC chemokine that was first discovered in 1987 by several laboratories as neutrophil chemotactic activity in the supernatant of activated monocytes, and later as chemotactic for T lymphocytes as well (253-257). Several other cell types secrete CXCL8, including neutrophils, T lymphocytes, fibroblasts, keratinocytes, endothelial, epithelial and malignant cells (105;234;258-262). In unstimulated cells, CXCL8 levels are relatively low, however, its production is rapidly induced by a wide range of stimuli, including pro-inflammatory cytokines such as TNFα, and IL-1β, bacterial and viral products, cellular stresses such as osmotic shock and oxidant stress, and growth factors such as VEGF, HGF, EGF, insulin-growth factor I (IGF-I) and IGF-II (105;253;261;263-273). CXCL8 binds with high affinity to two distinct receptors, CXCR1 and CXCR2, which are expressed in several cell types, including neutrophils, monocytes, lymphocytes, ECs and malignant cells (262;274-277).

A)

CXCL	Pro-angiogenic	Anti-angiogenic
ELR+	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	
ELR-	CXCL12, CXCL16	CXCL4, CXCL9, CXCL10, CXCL11, CXCL13, CXCL14

B)

CXCR	CXCL
CXCR1	CXCL6, CXCL8
CXCR2	all ELR+ ligands
CXCR3	CXCL4, CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL11, CXCL12

Figure 1.3: The CXC chemokine ligands and their CXC receptors.

A) A table showing the ELR-containing (ELR+) and the non-ELR-containing (ELR-) CXCLs with respect to their role in either promoting or inhibiting angiogenesis.

B) A table showing the CXCRs and their respective ligands.

1.5.1.1. Regulation of CXCL8 gene expression

The gene encoding CXCL8 is located on human chromosome 4 and possesses four exons and three introns. The CXCL8 promoter possesses a single transcriptional initiation site with a consensus TATA box (278). Several studies have demonstrated that CXCL8 expression is regulated by differential activation and binding of inducible transcription factors to the CXCL8 promoter (106;265;279). The sequence encompassing nucleotides –1 to –133 within the 5' flanking region of the CXCL8 promoter appears to be essential and sufficient for the transcriptional regulation of the gene (105;280-282). This region of the promoter contains binding sites for the transcription factors AP-1, CCAAT/enhancer binding protein (C/EBP-β or NF-IL6) and NFκB (105;281-284). The AP-1 binding site is situated between nucleotides –120 and –126 and it binds protein homodimers of the Jun immediate-early gene family (cJun, JunB and JunD), Jun/Fos members (cFos, FosB, Fra-1 and Fra-2) or Jun/ATF2 heterodimers (285). The C/EBP-β/NF-IL6 binding site is found between nucleotides –81 and –92, and interacts with protein dimers of the C/EBP family (286). The NFκB binding site is located between the nucleotides –70 and –80 and binds protein dimers of the Rel/NFκB family of transcription factors (287). The close proximity of these elements on the CXCL8 promoter suggests possible cooperativity and synergism in the regulation of CXCL8 gene expression. In fact, it is the cell type and stimuli that dictate which transcription factor or combination of transcription factors is responsible for transactivating the CXCL8 promoter (288).

Although CXCL8 expression is regulated mainly at the transcriptional level, post-transcriptional control, namely enhanced mRNA stability, has also been described (289-291). In fact, relatively low levels of CXCL8 present in unstimulated cells are the result of both repressed transcription and very unstable mRNA. The relatively fast degradation of CXCL8 mRNA is mediated by adenine and uracil (AU)-rich *cis*-elements (ARE) present in its 3' untranslated region (292;293). Moreover, enhanced CXCL8 mRNA stability has been classically associated with activation of the p38 MAPK pathway (292;293).

1.5.1.2. Role of CXCL8 in ECs and in angiogenesis

Human ECs express both CXCR1 and CXCR2 receptors (274;277;294). The involvement of CXCR2 in promoting *in vivo* angiogenesis has been demonstrated using CXCR2 knockout mice that experience a delay in cutaneous wound healing and are unable to induce angiogenesis in response to CXCR2 ligands (250;295). Koch *et al.* were the first to demonstrate, in 1992, that recombinant human CXCL8, as well as CXCL8 derived from conditioned media of activated monocytes, is angiogenic both *in vitro* and *in vivo* (296). The pro-angiogenic role of CXCL8 depends on the presence of the ELR motif, as mutation in this sequence results in a protein with angiostatic potential (237). *In vitro*, recombinant human CXCL8 or endothelial-derived CXCL8 enhances HUVEC and human dermal microvascular EC (HMEC) proliferation, migration and organization into capillary tubes, induces production of MMP-2 and MMP-9, and inhibits apoptosis by upregulating the expression of the anti-apoptotic genes B-cell leukemia/lymphoma 2 (Bcl-2) and x_L protein (Bcl-x_L). These effects are abrogated when these ECs are treated with neutralizing antibodies against either CXCL8, CXCR1 or CXCR2 (274;294;297).

Not all ECs express both CXCL8 receptors. In fact, human intestinal microvascular ECs only express CXCR2 and the angiogenic effects of CXCL8 on these cells are mediated solely through this receptor (298). Interestingly, enhanced expression of endothelial CXCL8 by pro-inflammatory and permeability inducing stimuli, including thrombin, TNFα and VEGF, promotes neutrophil adherence on EC surfaces as well as transendothelial migration. These effects are inhibited when ECs are pre-treated with anti-inflammatory agents such as Ang-1 and TGFβ1 (271;299;300). Thus, the effect of CXCL8 on ECs, whether pro-angiogenic or pro-inflammatory, largely depends on the type of stimulus and the cellular microenvironment. In human ECs, CXCL8 is stored in Weibel-Palade bodies, where it is sequestered by von Willebrand Factor (vWF) (301-304).

Finally, owing to its pro-inflammatory and pro-angiogenic potentials, CXCL8 has also been associated with tumour progression and tumour metastasis in a wide range of human cancers, including glioblastomas, melanomas and lung, ovarian, prostate, colon, cervical and breast cancers (105;262;305-310).

1.6. The angiopoietin family

1.6.1. Structure and expression profile of angiopoietins

Ang-1, the first ligand of the tyrosine kinase receptor Tie-2, was first identified in 1996. Currently, the angiopoietin family of growth factors is comprised of four members, namely angiopoietins-1, -2, -3 and -4 (311-313). All angiopoietins share a common structure composed of a carboxy-terminal fibrinogen (FN)-like domain responsible for binding to the receptor, preceded by a linker peptide region, a coiled-coil domain that mediates oligomerization of the FN-like domains through disulfide bonds, and a short hydrophobic amino-terminal domain that serves secretory and superclustering functions by generating variably sized multimers (311;312;314-316) (Figure 1.4) . In addition to the full length Ang-1 (1.5kb) spanning 498 amino acid residues, three additional species of Ang-1 generated by alternative splicing (1.3kb, 0.9kb and 0.7kb) and spanning 367, 285, and 154 amino acid residues, respectively, have recently been identified and sequenced (317). The functions of these isoforms are still unclear; however, it has been shown that the 1.3kb and 0.9kb isoforms, partially devoid of the FN-like and coiled-coil domains, respectively, may act as dominant negative regulators of full length Ang-1 (317). Likewise, alternative splicing of the full length Ang-2 (496 amino acid residues) generates an Ang-2 isoform (443 residues) lacking part of the coiled-coil domain. Ang-2_{443} inhibits Ang-1 induced Tie-2 activation (318). Another Ang-2 splice variant has been identified in *Gallus sp.* and has been designated Ang-2B. In addition to lacking part of the coiled-coil domain, Ang-2B also lacks the secretory signal sequence (318).

Although both (full-length) Ang-1 and Ang-2 bind Tie-2 receptors on the same domains and with similar affinities, initial findings have demonstrated that Ang-2 only weakly phosphorylates Tie-2 receptors and competitively blocks Ang-1-mediated receptor phosphorylation (64;311;312). The exact mechanisms behind this differential Tie-2 receptor activation following stimulation with either Ang-1 or Ang-2 are not well understood. However the phenomenon might be explained by differences in their structures and expression profiles. Ang-2 is primarily produced by ECs themselves, whereas Ang-1 is mainly produced by pericytes, vascular SMCs, fibroblasts, thyrocytes

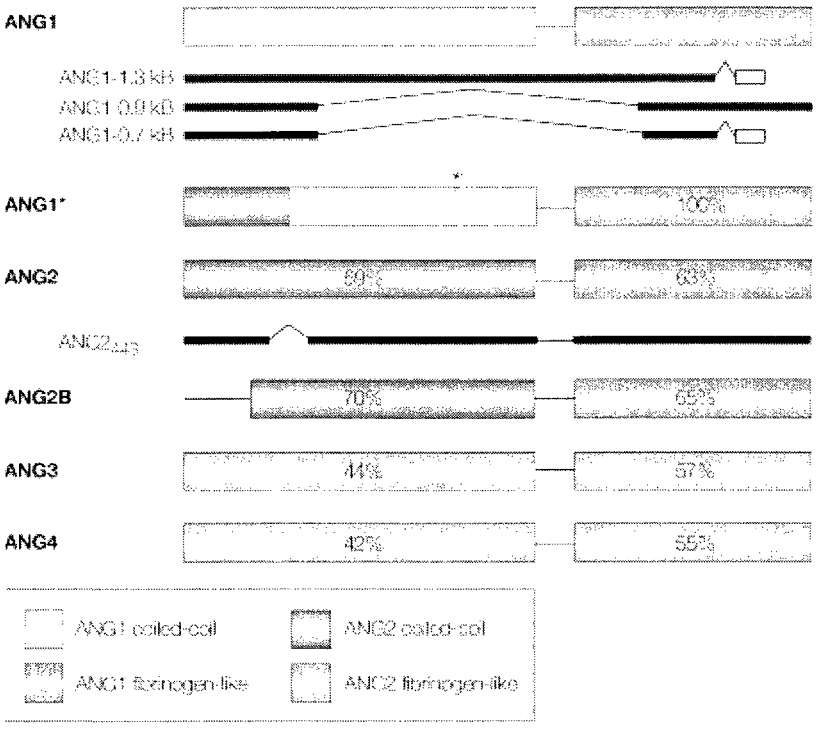

Figure 1.4: Domain structure of the angiopoietin family members.

% Amino acid identities with Ang-1. The asterisk represents a cysteine-to-serine substitution at amino acid residue 265 of the engineered version of Ang-1 (Ang-1*).

Jones N. *et al.* Nature Reviews Molecular Cell Biology 2: 257-267, 2001.

and adipocytes (75;78;311;312;319;320). Under physiological conditions in the human adult, Ang-1 mRNA is widely expressed in many tissues, whereas Ang-2 mRNA expression is mainly restricted to the ovaries, placenta and uterus (312).

Ang-1 and -2 also possess different properties in terms of interactions with the ECM. Ang-1 is incorporated into the matrix via its linker peptide region, whereas Ang-2 is secreted but does not interact with the matrix (321). In addition, Ang-2 can be stored in Weibel-Palade bodies in the cytoplasm of ECs, from where it can be rapidly released following appropriate stimulation (322). Finally, Ang-1, as a tetramer or higher-order multimer, produces maximal Tie-2 activation, whereas Ang-2 exists predominantly as a dimer (314;316;323). These findings suggest that limited physiological quantities of Ang-1, acting in a paracrine manner, are required for angiogenesis *in vivo*, as excess Ang-1 is retained in the ECM until free Tie-2 binding sites are available. To make matters more complex, availability is further controlled by autocrine effects of Ang-2 and recent reports have attributed a context-dependent agonistic function for Ang-2, whereby Ang-2 mediates some effects similar to those of Ang-1 (73;324;325). Recently, Ang-1 and Ang-2 have also been reported to bind integrins $\alpha_v\beta_5$ and β_1 *in vitro* with an affinity much less significant than that relating to Tie-2, suggesting that Ang-1 and Ang-2 bind integrins after saturating Tie-2 binding sites (326).

Murine Ang-3 and its human ortholog Ang-4 were identified as members of the angiopoietin family after their identification in 1999, based on structural similarities with Ang-1 and Ang-2 (313). Both Ang-3 and Ang-4 bind Tie-2 mainly as disulfide-linked dimers (43;313;327). As with Ang-2, Ang-3 competitively inhibits Ang-1 binding to, and subsequent phosphorylation of, Tie-2 (313). However, unlike Ang-1 and Ang-2, Ang-3 is tethered on the cell surface via interaction of its coiled-coil domain with heparan sulfate proteoglycans (HSPGs), especially perlecan. Excess amounts of Ang-3 are secreted in soluble form (327). It seems that this interaction is necessary for the bioactivity of Ang-3, since soluble Ang-3 is not capable of eliciting the same responses as that of the cell-bound form (327;328). Whereas Ang-4 appears to be mainly expressed in human lungs, a distribution similar to that of Tie-1, with unknown cellular sources, Ang-3 shows wide tissue distribution in mice and is produced by periendothelial support cells (313;327;329). Ang-4 stimulates phosphorylation of Tie-2 receptors on ECs, whereas Ang-3 only

activates Tie-2 in non-ECs and blocks Ang-1 induced Tie-2 activation in ECs. Based on these results, it has been suggested that Ang-3 and Ang-4 act, respectively, as an antagonist and agonist for endothelial Tie-2 (313).

Recently, the roles and mechanisms of actions of Ang-3 and Ang-4 have been compared both *in vitro* and *in vivo*. Results show that only Ang-4 induces activation of Tie-2 and protein kinase B (AKT) in HUVECs, promoting their survival and migration, whereas Ang-3 leads to more robust activation of Tie-2 and AKT in mice lungs than does Ang-4 (43). Thus, both Ang-3 and Ang-4 act as Tie-2 agonists in their respective species, and as with Ang-2, their functions seem to be context-dependent. This provides yet another example of differential regulation of Tie-2 receptors by different ligands, a regulation dictated by the cellular context.

1.6.2. Biological roles of angiopoietins during embryonic development

Ang-1 knockout mice display a phenotype similar to, but less severe, than that of mice lacking Tie-2 expression, characterized by severe vascular defects, lack of association of ECs with the underlying matrix and periendothelial cells and lethality by E12.5. This suggests that Ang-1, the main ligand for Tie-2 receptor, promotes vascular maturation and stabilization, at least in part, by recruiting periendothelial support cells (330). In comparison, transgenic mice over-expressing Ang-1 in the skin exhibit more vessels that are larger, more highly branched and resistant to vascular leakage, implying that Ang-1 promotes blood vessel branching and maturation and maintains vascular integrity (331;332). In addition, Ang-1 in combination with Tie-1 plays an important role in establishing vascular polarity during vascular development, as Ang-$1^{-/-}$Tie-$1^{-/-}$ double knockout mice show disorganized vessels in the right but not in the left hand side venous system at E8.5-E9.5 (333).

Thus far, contradictory findings have been obtained from studies aimed at elucidating the roles of Ang-2. It has been reported that overexpression of Ang-2 in mice leads to embryonic lethality at E9.5-E10.5 and to a phenotype similar to that of Ang-$1^{-/-}$ and Tie-$2^{-/-}$ mice, including immature blood vessel formation (312). However, in mice lacking functional Ang-2 expression, the hyaloid vasculature fails to regress postnatally and they show defects in lymphatic vessel development (334). In addition, Ang-2

deficient neonate mice develop disorganized renal cortical peritubular capillaries (335). This suggests a role for Ang-2 during late embryogenesis as well as during postnatal angiogenesis and lymphangiogenesis and further confirms a context-dependent role for Ang-2.

Since Ang-4 is found only in humans and since no experimental knockout or transgenic mice for Ang-3 have been developped to date, it is difficult to understand the physiological role of these ligands in either embryonic or adult development. However, data from *in vitro* studies suggest that Ang-3 and Ang-4 might play similar roles in terms of angiogenesis as those of Ang-2 and Ang-1, respectively (43;313).

1.6.3. Biological roles of angiopoietins in the adult vasculature

1.6.3.1. Roles of angiopoietin-1 in the adult vasculature

1.6.3.1.1. Roles of angiopoietin-1 in *in vivo* angiogenesis

Investigators have used varying approaches to determine how Ang-1 modulates *in vivo* angiogenesis, including the use of recombinant proteins, adenoviruses, naked plasmids and transgenic animals. In developing mice, localized overexpression of Ang-1 in the liver elicits significant enlargement and sprouting in hepatic arterial circulation and induces portal vein dilation (336). In adult mice, overexpression of Ang-1 in the skin results in a higher number of vessels that are larger, more highly branched and resistant to leakage induced by treatment with inflammatory agents (331;332). Similarly, delivery of a potent variant of Ang-1, cartilage oligomeric matrix protein (COMP)-Ang-1 using an adenoviral vector or recombinant protein produces enlarged blood vessels and enhanced blood flow in the tracheal microvasculature and promotes closure of dermal wounds in diabetic mice through increased angiogenesis and lymphangiogenesis (337;338). In addition, gene delivery of Ang-1 using plasmid DNA enhances collateral vessel formation in the rabbit ischemic hindlimb model (339;340). All of these studies confirm the crucial role of Ang-1 in promoting *in vivo* angiogenesis.

1.6.3.1.2. Roles of angiopoietin-1 in *in vitro* angiogenesis

Angiogenesis is the culmination of several processes that are designed to elicit degradation of basement membranes, proliferation, migration, adhesion and remodelling of ECs, eventually resulting in neovascularization. Using *in vitro* reductionist models, numerous investigators have studied how angiopoietins alter angiogenesis-related processes. Among the best-characterized effects of Ang-1 is its ability to inhibit apoptosis triggered by several stimuli. Results from our laboratory, as well as other laboratories, have shown that in response to serum withdrawal or TNFα, anti-apoptotic effects of Ang-1 in ECs are mediated through activation of both the phosphoinositide 3-kinase (PI3-KINASE)/AKT and the ERK1/2 MAPK pathways downstream from Tie-2 receptors. This leads to inhibition of the activity of caspases-9, -7 and -3, upregulation of the expression of the survivin protein and inhibition of second mitochondrial activator of caspase (Smac) release into the cytosol (341-344). The enhanced EC survival and vessel stabilization mediated by Ang-1 are due, at least in part, to the inhibition of forkhead transcription factor FOXO-1. In fact, FOXO-1 regulates the transcription of several genes involved in matrix remodelling and vessel formation, including Ang-2 (345).

Ang-1 also functions as a chemoattractant to promote EC migration in a Tie-2 dependent manner (319;346-348). Both the downstream-of-kinase-related (Dok-R) and PI3-kinase pathways are involved in Ang-1-induced EC motility and cytoskeletal reorganization. Recruitment of Dok-R through its phosphotyrosine binding (PTB) domain to Tyr^{1106} of Tie-2 leads to its phosphorylation, creating binding sites for additional molecules involved in EC migration, namely p21-activated kinase 1 (PAK1) and the adaptor protein Nck (349;350). Ras homolog gene family, member A (RhoA) and Ras-related C3 botulinum toxin substrate 1 (Rac-1), both GTPases, and endothelial nitric oxide synthase (eNOS), which are activated downstream from the PI3-kinase pathway, are also involved in enhanced EC migration in response to Ang-1 (347;348;351). In addition, the adaptor protein ShcA, through its SH2 domain, also interacts with activated Tie-2 receptor at Tyr^{1101} and expression of a dominant-negative form of ShcA abrogates Ang-1-induced EC motility (352). Other cellular effects in which Ang-1 is involved include EC sprouting and differentiation into tube-like structures in 2D and 3D matrices. Signalling intermediates implicated in these effects include PI3-kinase, ShcA, focal

adhesion kinase (FAK) and endothelial nitric oxide synthase (eNOS) (348;351-354). In line with these remodelling effects, Ang-1 induces the secretion of proteases, such as plasmin and matrix metalloproteases-2 and -9, and inhibits the production of tissue inhibitor of metalloprotease-2 (TIMP-2) (339;354;355). Although initial reports have shown either weak or no activation of EC proliferation by Ang-1, more recent studies, including our own employing HUVECs transduced with a retroviral vector expressing Ang-1, have revealed a moderate increase in EC proliferation (319;346;353;356-358).

1.6.3.1.3. Roles of angiopoietin-1 in inflammation

One of the major functions of the EC lining is to ensure the semi-permeability of the blood vessels, controlling the passage of solutes and inflammatory cells from the circulation to the underlying tissues. Studies have shown that Ang-1 exerts anti-inflammatory actions in ECs. *In vivo* and *in vitro* studies employing Ang-1 adenoviruses, overexpression of Ang-1 in transgenic mice or recombinant protein have demonstrated that Ang-1 protects against vascular leakage induced by inflammatory agents and cytokines (332;359;360). The mechanisms behind this anti-inflammatory action of Ang-1 have been well explored. It has been shown that Ang-1 strengthens the adhesion between ECs by localizing platelet endothelial cell adhesion molecule-1 (PECAM-1) to interendothelial cell junctions and decreasing the phosphorylation of PECAM-1 and vascular endothelial cadherin (359).

In venules, Ang-1 reduces plasma leakage by decreasing the number and size of endothelial gaps (361). Ang-1 counteracts the permeability enhancing effects of thrombin in ECs by inhibiting thrombin-induced Ca^{2+} flux and activation of protein kinase C zeta (Prkcz) and the transcription termination factor Rho (362). In addition, Ang-1 decreases endothelial permeability and the adhesion of leukocytes on VEGF-activated endothelium by several mechanisms. These include reducing the expression of adhesion molecules induced by VEGF, namely ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, preventing Ca^{2+} influx from the extracellular milieu and inhibiting Rous sarcoma oncogene (Src) activation by VEGF, an event needed for the phosphorylation-dependent internalization of VE-cadherin and disruption of interendothelial adherens junction (AJ) (363-365).

Furthermore, Ang-1 activates sphingosine kinase-1 (SK-1), the product of which, sphingosine-1-phosphate (S1P), acts on its receptors expressed on EC and inhibits EC permeability (366;367). Moreover, induced expression of tissue factor (TF), a pro-thrombotic and pro-inflammatory cytokine, by TNFα and VEGF is reduced by Ang-1 treatment (368). In fact, a decrease in the expression of Ang-1 by lipopolysaccharides (LPS) in the lungs is the mechanism behind the vascular leakage produced in a mouse model of acute lung injury (ALI) (369). In support of these findings, cell-based Ang-1 gene therapy has recently been shown to improve the indices of inflammation and lung injury in both rat and mouse models of ALI (370-372). In addition, COMP-Ang-1, a more potent form of Ang-1, attenuates bronchial inflammation, hyper-responsiveness and vascular leakage in asthmatic airways (373). These *in vivo* protective effects of Ang-1 require the activation (i.e. phosphorylation) of p190RhoGAP, a GTPase regulatory protein activated downstream from the GTPase, Rac1 (374).

Collectively, these data support an anti-inflammatory role for Ang-1. However, results from other laboratories support a pro-inflammatory role for Ang-1, because of an increase in the adhesiveness and adhesion properties of ECs and neutrophils, respectively. On the one hand, stimulation of ECs with Ang-1 induces P-selectin translocation from Weibel-Palade bodies (WPB) to the cell surface (73;375;376). P-selectin then interacts with its receptors on neutrophils and promotes rolling and transient adhesion on ECs (73;377). On the other hand, treatment of neutrophils with Ang-1 leads to both an induction of platelet-activating factor (PAF) synthesis and an upregulation of β_2 integrin, which interact with ICAM-1 and ICAM-2 on ECs. This leads to enhanced adhesion of neutrophils on ECs (73). These discrepancies could be at least partially explained by differences in the protocols used. The pro-inflammatory role of Ang-1 was detected shortly (minutes) after treatment of the cells with the growth factor, whereas the anti-inflammatory effects were seen after prolonged exposure to Ang-1 (overexpression in transgenic mice, adenoviral mediated gene transfer or several hours of exposure to recombinant protein).

1.6.3.2. Roles of angiopoietin-2 in the adult vasculature

Angiopoietin-2 was first identified as a naturally occurring antagonist of Ang-1, inhibiting Ang-1-induced Tie-2 phosphorylation, and therefore regulating Ang-1 mediated effects on ECs. The phenotype of the transgenic mouse overexpressing Ang-2 resembles that of Ang-1 and Tie-2 deficient mice (312). Angiopoietin-2, in contrast to Angiopoietin-1, destabilizes blood vessels, promotes vascular leakage and, in the absence of other vascular growth factors like VEGF, enhances vascular regression and EC apoptosis (312;378;379). However, under some conditions, Ang-2 has been found to act as a Tie-2 agonist. Therefore, the current paradigm holds that the effects of Ang-2 are context- and dose-dependent. The presence or absence of VEGF or Tie-1, the cell type or the vascular bed involved and the stage of maturation of the vessels in question are among the factors that define the context. It has been shown that as the vessels mature, they lose their sensitivity towards Ang-2 (380). Moreover, in the lymphatic vessels, Ang-2 activates Tie-2 receptors and is required for lymphatic patterning and maturation during development (334).

A controversy still exists as to whether Ang-2 induces phosphorylation and activation of Tie-2 outside the lymphatic system. Studies performed in several laboratories have shown that Ang-2 is able to stimulate Tie-2 phosphorylation only at high concentrations or following prolonged exposure, leading to enhanced EC survival, differentiation and mild Tie-2 receptor internalization (324;356;381). In a study performed in our laboratory, our group was able to demonstrate that Ang-2, even at physiological concentrations ($\leq$ 300ng/ml), is able to stimulate, although to a lesser extent than Ang-1, the phosphorylation of Tie-2, AKT, ERK1/2 and p38 and to inhibit the phosphorylation of JNK in HUVECs. Under such conditions, Ang-2 is able to inhibit EC apoptosis and promote EC survival (325). However, in another study, it has been shown that Ang-2 at low concentrations promotes angiogenesis and induces phosphorylation of Tie-2 and its downstream signalling molecules in human cord blood-derived EPCs but not in HUVECs. This has been demonstrated to be due to the presence of Tie-2 receptors unbound to Tie-1 in EPCs, whereas in HUVECs Tie-2 is found in a heterocomplex with Tie-1 (382).

Angiopoietin-2 expression is induced by several extracellular stimuli, including hypoxia and VEGF (383;384), and in patients with acute coronary syndromes or chronic heart failure (385;386). Owing to this fact, Ang-2 has the potential of being an important vascular growth factor involved in neovascularization at sites of vascular injury. Ang-2 is a major player in the initiation of retinal neovascularization (380). Overexpression of Ang-2 in the mouse retina increases the formation of new pericyte-deficient capillaries as well as induces the mRNA levels of VEGF and Ang-1 (387). In line with these findings, injection of angiopoietin-2 into the eyes of normal rats induces dose-dependent pericyte loss (388).

In addition to its role in angiogenesis, several studies have implicated Ang-2 in the promotion of inflammation. It has been shown that Ang-2 levels are increased in the plasma, alveolar edema fluid and tracheal aspirates of human adults and neonates with ALI. In mice exposed to high concentrations of oxygen ($\geq$ 50%), an inducer of ALI, mRNA and protein expression of Ang-2 in the lungs is induced whereas that of Ang-1 is reduced. This Ang-2 contributes to hyperoxia-induced epithelial cell death, DNA injury, oxidant injury, inflammation, edema and mortality (389). In addition, high levels of Ang-2 are found in the serum of patients with severe sepsis (390). Involvement of Ang-2 in inflammation has also been clearly demonstrated in Ang-$2^{-/-}$ mice. These mice fail to elicit an inflammatory response following a challenge with *Staphylococcus aureus*. However, recombinant Ang-2 restores the inflammation effect (391).

In cell cultures, Ang-2 sensitizes the endothelium to suboptimal concentrations of TNFα and enhances monocyte adhesion to the activated endothelium by further upregulating the expression of the adhesion molecules ICAM-1 and VCAM-1 (391). Ang-2 is stored in ECs in Weibel-Palade bodies and is rapidly released following stimulation (322). Its expression is inhibited in ECs by Ang-1 and shear stress in AKT-dependent inhibition of both expression and activation of the transcription factor FOXO-1 (345;392). Therefore, the balance between Ang-1 and Ang-2 is crucial in determining vessel stability and homeostasis.

1.6.3.3. Roles of angiopoietin-3 and angiopoietin-4 in the adult vasculature

The roles of Ang-3 and Ang-4 in the adult vasculature during both physiological and pathological angiogenesis are still unclear. In the mouse corneal assay, both Ang-3 and Ang-4 show pro-angiogenic activity, whereas Ang-4 alone induces EC migration and tube formation and inhibits EC increase in permeability *in vitro* (43;393). However, it has also been reported that overexpression of Ang-3 plays a role in inhibiting tumour angiogenesis by blocking EC proliferation and promoting EC apoptosis. These effects of Ang-3 are partly mediated by inhibition of Ang-1- and VEGF-induced activation of ERK1/2 and AKT kinase (328). In contrast, Ang-4 has been suggested as contributing to pulmonary vascular integrity in a murine model of LPS-induced acute lung injury, whereby Ang-4, like Ang-1, diminishes VEGF-induced inflammation (369). In addition, Ang-4 inhibits growth factor induced HUVEC migration, tumour angiogenesis and tumour vessel leakiness (394). Thus, it seems that Ang-3 and Ang-4 act as both inducers and inhibitors of angiogenesis. However, not much interest is being given to these molecules, as only a few studies have been directed toward understanding their roles in angiogenesis.

1.7. Tie-2 signalling

As with other RTKs, Tie-2 receptors possess an intrinsic tyrosine kinase activity. Major progress has been made in two areas of Ang-1/Tie-2 signalling, specifically, adaptor protein recruitments and the activation of upstream pathways, namely the PI3-kinase, the Dok-R and the Mitogen-activated protein kinases (MAPKs) pathways (341;343;344;395;396). However, little information is available about the nature of downstream modulators such as transcription factors and secreted mediators activated by the Ang-1/Tie-2 axis in ECs.

1.7.1. Adaptor proteins and phosphatases

Upon ligand stimulation, the receptor dimerizes and cross-phosphorylates multiple tyrosine residues in its cytoplasmic tail (397). These phosphorylated tyrosine residues create docking sites for several cytoplasmic signalling molecules containing

phosphotyrosine binding sites, such as the Src homology 2 (SH2) domain and, to a lesser extent, the PTB domain (398). These SH2 or PTB containing cytoplasmic targets have diverse biological and biochemical functions, as some possess intrinsic enzymatic activities, whereas others serve as scaffolds or adaptors for recruiting further downstream signalling molecules (397).

Several tyrosine residues are phosphorylated in the intracellular region of activated Tie-2 receptors. Phosphorylated mouse Tyr^{1100} serves as a multisubstrate docking site that is important for the recruitment of the growth factor receptor-bound proteins (Grb) 2 and 7, as well as the p85 subunit of PI3-kinase, through their SH2 domains, both *in vivo* and *in vitro*, whereas the adaptor protein Grb14 preferentially binds to Tyr^{814} and Tyr^{1106} (399). Dok-R binds to mouse Tyr^{1106} on activated Tie-2 receptors through a PTB domain both *in vitro* and *in vivo*, and also possesses a pleckstrin homology (PH) domain that can localize it to the plasma membrane and further enhance its binding to Tie-2, possibly in a PI3-kinase dependent fashion (350;396).

The SH2-containing tyrosine phosphatase-2 (SHP-2) associates with several phosphotyrosine residues on activated mouse Tie-2, including Tyr^{814} and Tyr^{1111} (399). It has been reported that occupancy of the SH2 domains of SHP2 enhances its phosphatase activity (400). In accordance with these findings, disruption of the association of SHP2 with Tie-2 by mutation or truncation of its binding site on the receptor, leads to increased Tie-2 autophosphorylation and enhanced activation of downstream signalling pathways (350;395;401). In addition to SHP-2, the vascular endothelial protein tyrosine phosphatase (VE-PTP), an EC-specific receptor type phosphotyrosine phosphatase, associates with activated mouse Tie-2 (402;403). Differential binding of these phosphatases to Tie-2 may play an important role in modulating the activity of the receptor.

In contrast to the situation in mice, activation of human Tie-2 receptors has been much less investigated. It has been reported that Grb2 and SHP2 may link receptor tyrosine kinase to AKT and MAPK signalling and associate respectively with Tyr^{1101} and Tyr^{1112} of human Tie-2 receptors (395;404-408). Furthermore, the p85 regulatory subunit of PI3-kinase associates with both Tyr^{1101}, and, to a much lesser extent, Tyr^{1112}, leading to the activation of PI3-kinase and AKT (395). In addition, the adaptor protein ShcA is

phosphorylated and recruited through its SH2 domain to Ang-1-activated Tie-2, an association involving Tyr^{1101} of Tie-2 and leading to EC sprouting and migration (352).

Activated Tie-2 receptors also trigger the production of reactive oxygen species (ROS), mainly H_2O_2, by way of NADPH oxidase (409;410). Such ROS production requires Rac-1 and activated downstream effectors such as AKT and MAPKs. ROS participate in Ang-1-induced EC migration and *in vivo* tubule formation. In addition, the novel protein, A20 binding inhibitor of NFκB activation-2 (ABIN-2), has been found to interact with phosphorylated Tie-2 receptors and to play a role in mediating the pro-survival and, possibly, the anti-inflammatory effects of Ang-1, through activation of the PI3-kinase/AKT pathway (411;412). The diversity of intracellular signalling molecules engaged downstream from activated Tie-2 receptors further suggests a role of Tie-2 as a molecular switch, depending on the cellular microenvironment.

1.7.2. Pathways activated by Ang-1/Tie-2 axis

According to the established scheme of Ang-1/Tie-2 signalling, three main pathways are activated immediately downstream of the adaptor proteins, namely, the PI3-kinase, the Dok-R and the MAPKs.

1.7.2.1. The phosphoinositide 3-kinase (PI3-kinase) pathway

PI3-kinases (also called phosphoinositide 3-OH kinases) constitute an ubiquitous family of lipid kinases that catalyze the addition of a phosphate group to the 3-hydroxyl position of the inositol ring of membrane-localized phosphatidylinositol (PtdIns), leading to the generation of 3'-phosphorylated phosphoinositides (PIs) (413;414). To date, nine mammalian PI3-kinases have been identified and categorized into three main classes (I, II, III), depending on their sequence homologies, lipid specificities and regulation (413;415). PI3-kinases are heterodimeric proteins that are composed of a catalytic subunit and a regulatory subunit (415;416). They generate four functionally different lipid products, namely PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-3,5-P_2 and PtdIns-3,4,5-P_3 (413). These PIs serve as docking sites for several signalling molecules that contain a PH domain and that perform a wide array of cellular functions, including migration, proliferation, inflammation, glucose metabolism and survival (417-422).

Class I enzymes predominantly phosphorylate cellular PtdIns-4,5-P_2, leading to the generation of PtdIns-3,4,5-P_3 in response to stimulation by growth factors (414;423). They are generally composed of a p110 subunit that contains a lipid-directed catalytic domain and a p85 regulatory subunit that recruits, through interaction of its SH2 domains with the receptor's phosphotyrosines, the catalytic subunit to activated tyrosine kinase receptors (415). In addition to their lipid kinase activity, class I PI3-kinases also possess protein kinase activity at sites of serine and threonine residues, employing as substrates either the catalytic subunit itself or the regulatory subunit. This results in downregulation of lipid kinase activity (424-427). Dephosphorylation of PtdIns-3,4,5-P_3 and termination of class I PI3-kinase signalling occurs through the action of two inositol lipid phosphatases: phosphoinositide-lipid 3-phosphatase (PTEN) and the SH2-containing inositol polyphosphate 5-phosphatase (SHIP) (428;429). Moreover, class I enzymes activate mammalian targets of rapamycin (mTOR)/S6kinase (S6K), which plays a role in the regulation of protein synthesis, cell growth and insulin metabolism. Upstream activators of mTOR are not well characterized, however, it has been reported that activation can take place independently from serine/threonine kinase (AKT) (430-433). Class II PI3-kinases mainly phosphorylate cellular PtdIns-4-P to produce PtdIns-3,4-P_2 which is involved in promoting cell survival (413;414). Class III PI3-kinases are specific for the substrate PtdIns and generate the majority of PtdIns-3-P in mammalian cells, which plays a role in vesicular trafficking and endocytosis (414;434-436).

Signalling molecules containing a PH domain accumulate at sites of PI3-kinase activation, in particular serine/threonine kinase AKT-1/PKBα/RAC-PKα (protein kinase B/related to A and C protein kinase), which promotes cell survival, and the phosphoinositide-dependent kinase 1 (PDK1) (421;422). The mammalian AKT family has two additional members, AKT-2/PKBβ/RAC-PKβ and AKT-3/RAC-PKγ, playing respective roles in the promotion of glucose uptake and vascular SMC proliferation (437;438). Following survival stimuli, AKT translocates to the plasma membrane through association with PtdIns-3,4,5-P_3 then undergoes a conformational change that unmasks serine and threonine phosphorylation sites and facilitates activation of AKT by PDK1/protein kinase C-related kinase-2 (PDK-1/PRK-2) (421;439;440). AKT is a multifunctional protein kinase that is involved in the regulation of several cellular

responses (441). It is well documented that AKT plays a major role in PI3-kinase-mediated cell survival in various cell types and in response to different apoptotic stimuli, including growth factor withdrawal, DNA damage and UV irradiation, through either posttranslational or transcriptional mechanisms (421;442-447).

Several downstream targets of AKT are also known to be involved in apoptosis, including bcl2-associated death promoter (Bad), caspase-9, inhibitors of apoptosis (IAPs), Smac/DIABLO, inhibitory kappa kinases (IKK) α and β and the FKHR family of transcription factors (342;448-452). In addition to its role in cell survival, AKT increases eNOS activity, stimulates glucose uptake by inducing the translocation of the glucose transporter GLUT4 to the cell membrane, enhances glycogen synthesis by phosphorylating and inhibiting glycogen synthase kinase-3 and promotes protein synthesis by phosphorylating and activating mTOR, ribosomal S6K and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (453-458). Of particular note is that improper activation of the PI3-kinase/AKT pathway contributes to several pathologies, including carcinomas and inflammation (459-461).

1.7.2.1.1. Regulation of endothelial cell survival by the angiopoietins/Tie-2 and the PI3-kinase/AKT pathway

One of the best-characterized cellular functions of the Ang-1/Tie-2 pathway is its involvement in promoting EC survival, although very few studies have been performed regarding the roles of Ang-2 and Ang-4 in cell survival. The first report demonstrating the activation of the PI3-kinase/AKT pathway downstream from Tie-2 receptors was published in 1998, where it was shown that the p85 regulatory subunit of PI3-kinase associates with phosphorylated human Tie-2 receptors at Tyr^{1101} and, to a lesser extent at Tyr^{1112}, leading to AKT activation (395). Subsequently, two other laboratories implicated Ang-1 and Tie-2 in EC survival, as downregulation of Tie-2 expression leads to EC death, and treatment with Ang-1 inhibits serum-withdrawal induced apoptosis (462;463). Additional studies correlated EC survival with the activation of the PI3-kinase/AKT pathway downstream from Ang-1 and implicated the upregulation of the pro-survival protein survivin as one of the underlying mechanisms (343;344). In addition, our group and others have shown that Ang-1 activates the PI3-kinase/mTOR/S6K pathway. This

activation results in enhanced EC proliferation, prevention of iopromide (a reagent used in X-ray analysis)-induced EC death and upregulation of the expression of pro-angiogenic genes such as VEGF-C, Egr-1 and Angiopoietin-like 4 (177;357;464). Moreover, our group was the first to show that the pro-survival effects of Ang-1/Tie-2 in HUVECs are mediated through the activation of PI3-kinase/AKT, inhibition of Smac release from the mitochondria, induction of survivin protein, and inhibition of caspase 3, 7 and 9 activities (342).

Although major advances have been made regarding the role of the PI3-kinase/AKT pathway in Ang-1/Tie-2 signalling, it is not yet clear whether or not other angiopoietins also activate this pathway in the event of EC apoptosis. Studies have shown that high concentrations of Ang-2 (800ng/ml or 2μg/ml) or long-term exposure to Ang-2 are capable of activating Tie-2, PI3-kinase, AKT and S6K, leading to EC survival following serum-induced apoptosis and without any effect on EC proliferation (324;357;465). However, at high concentrations, Ang-2 is also capable of binding to integrins, suggesting that the anti-apoptotic effect is due to both Tie-2 activation and integrin ligation (326;466). In addition, Ang-2 expression is highly modulated downstream from the PI3-kinase/AKT pathway in ECs treated with Ang-1 or VEGF, suggesting that a negative feedback mechanism is operating to maintain a high Ang-1/VEGF:Ang-2 ratio (345;467).

1.7.2.2. The downstream-of-kinase-related (Dok-R) pathway

The Dumont lab first identified Dok-R, co-expressed with Tie-2 in a number of EC lines, as a novel Dok-related docking protein involved in Tie-2 signalling. Dok-R contains an amino-terminal PH domain, possibly involved in translocation to the plasma membrane following Tie-2 activation, a central PTB domain that is required for binding to activated Tie-2, six proline rich residues (PXXP) that are essential for the recruitment of SH3 containing molecules, as well as a total of thirteen tyrosine residues that may potentially act as docking sites (396). This suggests that Dok-R might play an important role in further amplifying signalling downstream from Tie-2.

Dok-R is recruited to Tyr^{1106} on activated Tie-2 receptors through its PTB domain and is then tyrosine phosphorylated. This phosphorylation creates high affinity binding

sites for additional signalling molecules involved in EC migration and cytoskeletal reorganization, including PAK1, the receptor tyrosine kinase c-Abl, and the adaptor protein Nck (349;350;396;468). In addition, Dok-R recruits the enzyme rasGAP, a GTPase activating protein that functions as a negative regulator of Ras signalling, leading to the attenuation of ERK1/2 activation (396;469). Dok-R also reduces ERK1/2 and AKT activity, independently from rasGAP, through the recruitment of the pro-migratory kinase c-Src and the negative regulator of Src, C-src tyrosine kinase (Csk) (470). Other than in EC migration, functional roles of Dok-R in biological processes associated with the Ang-1/Tie-2 axis remain unclear. However, the aforementioned studies indicate that Dok-R might function as a molecular switch tilting the balance towards EC migration versus survival and proliferation.

1.7.2.3. The mitogen-activated protein kinase (MAPK) pathway

MAPKs are serine/threonine kinases. The most fully-characterized MAPK family members include ERK1/2, SAPKs/JNKs and the p38 MAPKs. Other family members that have been identified are BMK-1 (Big Map Kinase 1)/ERK5, ERK6/p38 gamma and ERK7, however their functions remain unclear (471;472). MAPKs can be further subdivided according to their TXY activation motif. ERK1/2, ERK5 and ERK7 possess the TEY (Thr-Glu-Tyr) motif, SAPKs/JNKs contain the TPY (Thr-Pro-Tyr) motif, whereas the TGY (Thr-Gly-Tyr) motif is found on p38 MAPKs and ERK6 (471).

The classical MAPK pathway allows the sequential activation of MAPK/ERK kinase kinase (MEKK), MAPK/ERK kinase (MEK) and MAPK, thereby linking various cell surface receptors with intracellular targets (473). This highly conserved pathway is initiated by the translocation of guanine nucleotide exchange factors (GEFs) to the plasma membrane where they activate the Ras GTPases by catalyzing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on Ras (474-476). GEF translocation to the plasma membrane is essentially facilitated by SH2 domain-containing adapter proteins, which associate with phosphotyrosine binding sites on activated receptors or receptor substrates (397;477).

In mammals, fourteen MEKKs and seven MEKs have been characterized to date (478). MEKKs comprise a family of serine/threonine kinases that are activated following

their translocation from the cytosol to the plasma membrane after binding to small GTPases (479). MEKs possess dual specificity and can phosphorylate both threonine and tyrosine residues on MAPKs (479). The specific steps in this cascade include the recognition of MAPKs by their cognate MEKs, protein tyrosine phosphatases (PTPs) and dual specificity protein tyrosine phosphatases (DSPs). MAPK signalling can be terminated by dephosphorylation of specific tyrosine and/or threonine residues mediated by PTPs and DSPs (478). MAPK signalling can also be inhibited at the level of Ras GTPases by GTPase activating proteins (GAPs), which enhance the intrinsic GTPase activity of Ras, thus bringing back Ras GTPases into their inactive (GDP-bound) state (476). Upon activation of MAPKs, they translocate to the nucleus and activate a wide range of transcription factors, primarily of the Ets family (479;480).

The Ets family consists of over fifty transcription factors, with the major targets including immediate early genes such as JunB, c-fos and Egf-1, in addition to a broad variety of endothelial specific genes involved in vasculogenesis, angiogenesis, growth and survival, such as Tie-1 and –2, Ang-1 and Ang-2, VEGFR-1 and –2, cyclin D and Bcl-2 (481-487). Based on this, it is possible that distinct Ets members might represent some of the unidentified transcription factors binding Oct-1, hence mediating Tie-1 and Tie-2 transactivation during embryonic development (69;488). Other MAPK targets include several nontranscriptional cytoplasmic substrates, such as growth factor receptors, and other kinases and proteins involved in apoptosis (479;489). The large number of players involved in MAPK signalling pathways and numerous crosstalk interactions between these pathways allow cells to quickly and effectively fine-tune their responses, so as to adapt to their microenvironments.

1.7.2.3.1. Biological roles of MAPKs

ERK1 and 2 (also referred to as p44 and p42) are ubiquitously expressed MAPKs with 90% sequence homology that display functional redundancy (479). They are mostly activated by mitogens downstream of RTKs, but can be activated as well downstream of G protein-coupled receptors (GPCRs) (490). ERK1/2 mediate cellular growth and transformation, cytoskeletal reorganization, differentiation and survival, with their spatiotemporal regulation often dictating the type of response obtained (473;491). For

instance, in pheochromocytoma and neuronal cells, sustained activation of ERK1/2 correlates with cell differentiation or apoptosis, whereas transient activation results in cell proliferation (492;493). Following RTK activation, the adapter protein Grb-2, which is constitutively bound to the GEF Son-of-sevenless (Sos), is recruited to the plasma membrane, where this complex activates Ras GTPase at the plasma membrane (477;494). Sos mediates the exchange of GDP to GTP on Ras thus creating a binding site for Raf-1 (a MEKK), leading to Raf-1 activation and a subsequent phosphorylation cascade composed of MEK1/2 and ERK1/2 (477;494). There are over fifty ERK1/2 substrates suggestive of the various functions of this kinase family (495). The majority of substrates are transcription factors involved in cell proliferation, including Ets, Elk-1, c-Fos and c-Myc, as well as cytosolic substrates such as Sos, MEK and cytoskeletal components (477;479;496-500).

Contrary to ERK1/2, SAPK/JNK and p38 MAPKs are weakly activated by growth factors and are mainly induced by a variety of stresses including heat and osmotic shock, reactive oxygen (ROS) and nitrogen (NOS) species generated by DNA damaging agents, ultraviolet light and inflammatory cytokines (501-504). These kinases are mainly activated downstream of Ras-homologous (Rho) family members, specifically Rac1/2 and Cdc-42, which are powerful migration inducers (474;505;506). Selective MEKs upstream of SAPK/JNK and p38 MAPKs include MKK-4/SEK-1 and MKK-7, and MKK-3 and MKK-6, respectively (503).

The SAPK/JNK family includes eight to ten isoforms that result from the alternative splicing of three genes: JNK1, 2 and 3 (503;504). Whereas JNK1 and JNK2 are ubiquitously expressed, JNK3 has been found to be primarily present in brain, heart and testis (503). c-Jun is the most well-characterized SAPK/JNK transcriptional target, however, SAPK/JNK have also been shown to activate a large number of transcription factors such as activating transcription factor 2 (ATF2) and Elk-1, suggestive of its diverse functions. Four genes encode the p38 MAPK family, including p38α, β, γ (SAPK3/ERK6) and δ (SAPK4) (507;508). All p38 mRNAs are ubiquitously distributed, apart from the γ isoform which is primarily present in the skeletal muscle (509). p38 MAPKs mediate actin reorganization and consequent cell migration at the onset of angiogenesis. Some differences exist between α and β isoforms. Whereas p38β is

preferentially activated by MKK-6, p38α shows no selectivity between MKK-3 and MKK-6 (510). In addition, whereas p38β more potently activates the pro-inflammatory transcription factor, ATF2, both p38 α and β recognize common substrates, including the transcription factors MEF2A and C, which contribute to c-Jun activation, and the small heat shock protein hsp27 (511;512). Hence, p38 MAPK isoforms have divergent yet overlapping roles, allowing a cell to preferentially rely on one or the other isoform, according to the microenvironment and external cues.

1.7.2.3.2. Biological roles of MAPKs in ECs

When our laboratory started to study the roles of MAPK in angiopoietin-1/Tie-2 signalling, there was only one report published showing that exposure of ECs to Ang-1 induces transient phosphorylation of ERK1 and 2 in ECs (346). However, the physiological significance of this phosphorylation and the mechanisms regulating ERK1/2 activation in response to Ang-1 remained unclear at that time. A better understanding of the biological roles and regulation of MAPKs family members in EC function comes from studies of the VEGF receptor signalling. It has been reported by several groups that VEGFs activate both the ERK1/2 and p38 MAPKs pathways in various ECs, including microvascular (capillary) and large vessel (umbilical vein and aorta) ECs, resulting in EC proliferation and survival, or migration, respectively (513-515). The roles of SAPK/JNK in VEGF signalling are particular in that it depends on cellular context. For example, during ceramide or serum-withdrawal-induced apoptosis, VEGF-dependant survival occurs as a result of simultaneous activation of ERK1/2 and inhibition of SAPK/JNK in human dermal microvascular ECs, however, in microvascular bovine ECs, VEGF-mediated EC proliferation results from simultaneous activation of SAPK/JNK and ERK1/2 (516;517).

Generally, activation of the ERK1/2 pathway has been shown to attenuate apoptosis (518-520). The roles of SAPK/JNK and p38 MAPKs in cell survival are still not clear, and despite the fact that they promote apoptosis in various cell types, they have also been shown to induce cell survival. Apparently, this depends on the duration and intensity of their activation, the cell type, and cellular context (503;520;521). In HUVECs, activation of ERK1/2 has been reported to inhibit serum deprivation-induced

apoptosis by phosphorylating and activating the anti-apoptotic protein Bcl-2 and to promote cell proliferation (357;522). Work performed in our laboratory using ECs demonstrated that Ang-1 simultaneously activates two MAPK pathways with opposing effects, the anti-apoptotic ERK1/2 and the pro-apoptotic p38 pathways. However, since Ang-1 induces stronger ERK activation than p38 activation, the net effect of Ang-1 is to promote EC survival (341). In line with our findings, one report showed that apoptosis occurs following caspase-3 activation by p38 MAPKs downstream of ROS in HUVEC (523). In contrast, it has been demonstrated that inhibition of p38α by protein tyrosine phosphatases, due to adenosine and/or homocysteine stresses, results in EC apoptosis (524).

Thus, it appears that ERK1/2 activation is a general inhibitor of apoptosis and promoter of cell proliferation, therefore, the dual roles of the SAPK/JNK and p38 MAPKs in the regulation of cellular apoptosis are stimulus- and cell type-specific. *In vivo*, these variables provide the cells with the ability to fine-tune their responses to the plethora of environmental cues they receive simultaneously, resulting in tissue-specific responses.

1.7.2.3.3. Interactions between MAPKs

Several studies provide evidence showing that after RTK activation, cells integrate the signals of various simultaneously activated MAPK family members before eliciting a biological response. This integration is mediated by complex interactions among members of the MAPK family, possessing similar or opposing functions in processes such as proliferation, migration and apoptosis. For example, both pro- and anti-apoptotic MAPK family members are activated in ECs upon VEGF stimulation (525). It has been suggested that the final cellular biological response relies on the relative level of activation of each MAPK pathway regulated, in turn, by positive and/or negative interactions among these pathways (519;526). The point of convergence can take place upstream, downstream or within the pathways in question (473). Proposed molecular switches include the MEK kinase kinase kinases (MEKKKs) which are activated downstream of the Ras superfamily of GTPases and are able to activate ERK1/2, p38 MAPKs and SAPK/JNK individually or simultaneously (507;527). The Ras superfamily

has also been shown to act as a molecular switch, mainly with respect to Ras, Rac and Cdc42 (474;475;528;529).

Negative interactions have been reported to occur among members of the ERK1/2 and p38 MAPK pathways, as well as between ERK1/2 and the SAPK/JNK members. It has been shown that membrane blebbing (a hallmark of apoptosis) and p38β MAPK activation induced by oxidative stress become amplified upon ERK2 inhibition in ECs and fibroblasts. This suggests that ERK2 plays a role in cell survival in these cells, as opposed to p38β, which seems to act in a pro-apoptotic manner (530). Moreover negative crosstalk has also been observed between the ERK1/2 and p38 MAPKs during ischemia/reperfusion in neonatal rat cardiomyocytes where ERK1/2, p38 MAPKs and SAPK/JNK get simultaneously activated (531). Inhibiting the ERK1/2 pathway in these cells using the MEK1/2 inhibitor PD98059 leads to enhanced apoptosis and augmented activation of both p38 MAPKs and SAPK/JNK pathways, demonstrating the ability of these cells to switch from survival (ERK1/2) to apoptosis (p38 MAPKs and SAPK/JNKs) depending on the relative activation of each pathway (531).

In contrast, a few studies have demonstrated positive interactions between the ERK1/2 and p38 MAPK pathways. In keeping with this, activity of p38 MAPKs upstream of the ERK1/2 pathway results in the synthesis of thrombin-induced prostacyclin (a vasodilator) in ECs (532). Moreover, ERK1/2 and p38 are more likely to be activated than the SAPK/JNK pathway during vasoconstriction in arthritis animal models and pathogen-induced cardiac apoptosis (533-535). In the arthritis model, inhibition of ERK1/2 has been demonstrated downstream of SAPK/JNK (534). Thus during inflammation, ERK1/2 phosphorylation can cooperate with p38 MAPKs to induce inflammation, possibly by promoting inflammatory cell survival (534). Another report accentuating the context-dependency regulating p38 MAPKs and ERK1/2 crosstalk has shown that p38 MAPKs promote ERK1/2-induced ECM assembly upon mechanical, but not growth factor, stimulation (536).

Whereas the interactions between the ERK1/2 and p38 MAPKs are widely documented, there are fewer reports showing interactions between the ERK1/2 and SAPK/JNK pathways. Positive interactions between both pathways have been shown following VEGF-induced proliferation of bovine aortic macrovascular ECs, where

SAPK/JNK activation was observed downstream of ERK1/2 (517). Negative interactions have also been reported upon VEGF stimulation of human dermal microvascular ECs. In one case, VEGF led to the inhibition of ceramide and serum deprivation-induced apoptosis through the simultaneous activation of the ERK1/2 pathway and inhibition of the SAPK/JNK pathway (516).

The mechanisms regulating the interactions between the ERK1/2 pathway and the p38 MAPKs and SAPK/JNK pathways still need to be widely explored. Possibly the dual specificity protein tyrosine phosphatase MKP-1 is involved since it selectively dephosphorylates and inhibits the p38 MAPKs and SAPK/JNK pathways, although not the ERK1/2 pathway (537). Another possible means of negative crosstalk involves caspase-3 and –7 which can cleave the N-terminal of MEKKs, thus differentially activating downstream kinases, including SAPK/JNK and ERK1/2 (538;539). Another factor, known as dual-specificity phosphatase 2 (DUSP2/PAC1), binds ERK1/2, p38 MAPKs and SAPK/JNK. DUSP2/PAC1 preferentially activates ERK1/2 and p38 MAPKs over SAPK/JNK (534). This activation is mediated by targeting different phosphorylation sites on MAPKs and by MAPK sequestration to selective subcellular localizations (534). Hence, it has been suggested that the net effects of these pathways are highly dependent upon the duration and the level of activation of individual MAPK pathways.

1.8. Role of Tie-1 and integrins in mediating angiopoietin intracellular signalling in ECs

Tie-1, the second member of the Tie subfamily of EC specific tyrosine kinase receptors, was considered until recently an orphan receptor. Although not much is known about the activation of the Tie-1 receptor, the downstream signalling events and the ligands involved, genetic experiments in mice deficient in Tie-1 have revealed a crucial role of the latter in promoting vascular maintenance and integrity and EC survival. These embryos exhibit a lethal phenotype as they die between E13.5 and birth due to severe haemorrhages and edema, suggesting that Tie-1 might be required later than Tie-2 during embryonic development (13;88). In addition, studies in chimeric embryos have revealed

that Tie-1 and Tie-2 have distinct non-redundant functions and that Tie-1 is required not only during embryonic vessel development but also in the maintenance of a quiescent adult vasculature (540). Interestingly, Tie-1 expression is induced in the same conditions and by the same factors that induce Ang-2 expression, namely hypoxia, VEGF, wound healing, ovarian follicle maturation and tumour angiogenesis (541-543). In terms of overall amino acid sequence, Tie-1 and Tie-2 are 44% identical. This similarity increases to 78-84 % at the level of the intracellular kinase domains and decreases to about 22-30 % at the level of the extracellular immunoglobulin-like ligand-binding domains, probably explaining why angiopoietins bind more potently to Tie-2 than to Tie-1 (63). The extracellular domain of Tie-1 can be proteolytically cleaved and subsequently released to the extracellular space following activation of PKC, treatment with VEGF or TNFα or by exposure to shear stress in a mechanism that possibly involves MMP activation (544-548). The remaining C-terminal endodomain is unphosphorylated and forms a complex with Tie-2, thus serving as a modulator of Tie-2 activation in ECs (65;547). This endodomain can associate with the phosphatase Shp2, indicating that Tie-1 might participate in a ligand-independent signalling pathway (549). More evidence that Tie-1 can modulate Tie-2 signalling and function is the finding that in HUVECs, Tie-1 forms a heterocomplex with Tie-2 thereby inhibiting Ang-2 induced Tie-2 phosphorylation (382). In addition, the Brindle laboratory has recently shown that proteolytic cleavage of the Tie-1 ectodomain is followed by enzymatic degradation of its endodomain and this leads to increased binding of Ang-1 to Tie-2 and enhanced Tie-2 activation (550).

Three studies thus far have shown that Tie-1 can be phosphorylated and can activate downstream signalling pathways. In the first study, Kontos *et al.* has shown that colony-stimulating factor 1 (CSF-1) is able to activate the c-fms-Tie-1 chimeric receptor, composed of the extracellular domain of CSF-1 receptor and the intracellular domain of Tie-1, stably transfected in mouse fibroblast cells leading to receptor autophosphorylation and inhibition of UV irradiation-induced apoptosis in a PI3 kinase and AKT dependent manner (551). However, these data could be the result of the non-EC microenvironment and/or an artefact of transfection. Whether these same mechanisms take place in ECs remains to be verified. In fact, the same phenomenon was seen in Ang-2-induced Tie-2 phosphorylation, which is significantly more powerful in mouse fibroblasts

overexpressing Tie-2 than in ECs naturally expressing Tie-2 (312). The second study, by Saharinen *et al.*, was the first to demonstrate that a signal can be transduced by full length Tie-1. COMP-Ang-1, a highly soluble form of Ang-1, as well as native Ang-1 and Ang-4 are able to induce Tie-1 phosphorylation in ECs and this activation is further enhanced by Tie-2 (552). These findings have recently been corroborated and expanded by Yuan *et al.* who have shown that Ang-1-induced Tie-1 phosphorylation is dependent on the kinase activity of Tie-2 and that Tie-1 activation downregulates Tie-2 mediated intracellular signalling and EC survival. These results help explain the increased capillary density in the Tie-1 null embryos (553). Given all the information available thus far, Tie-1 appears to function primarily as a modulator of Tie-2 signalling.

In addition to Tie receptors, Ang-1 and Ang-2 have the ability to bind different integrins. Ang-1 and Ang-2 in the matrix promote EC adhesion and ERK1/2 activation; both effects are blocked by RGD peptides (326). The adhesion of fibroblasts to Ang-1 and Ang-2 is mediated by $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins (326). The binding of Ang-1 to $\alpha_5\beta_1$ occurs through its fibrinogen-like receptor-binding domain (554). In skeletal and cardiac myocytes lacking the Tie-2 receptor, Ang-1 induces activation of AKT and ERK1/2 and promotes cell survival. RGD peptides and anti-integrin antibodies block these responses (466). Thus, angiopoietin-1 can signal through integrins in a Tie-2 independent manner. However, high concentrations of Ang-1 (10-100 μg/ml) are needed to induce integrin ligation.

1.9. Downstream effectors and modulation of gene expression downstream from the Ang-1/Tie-2 pathway

Little information is known regarding downstream effectors, such as transcription factors, activated by the Ang-1/Tie-2 signalling pathway. It has been shown that Ang-1 modulates the transcriptional activity of FOXO-1 by inhibiting the induction of its target genes involved in vessel destabilization and EC death (345). Another report has identified members of the signal transducers and activators of transcription (STATs), namely STAT3 and STAT5, as targets of Tie-2 activation in ECs. Such activation leads to enhanced expression of p21 mRNA, a cell cycle inhibitor involved in growth arrest and

cell death (555). STATs continuously shuttle from the cytosol to the nucleus and, upon activation, are retained in the nucleus where they modulate gene expression. Elk-1 is another transcription factor activated downstream from ERK1/2 by Ang-1. Elk-1 forms a ternary complex by binding SRF and SRE in various promoters (341). Finally, NERF2, an Ets family transcription factor, is induced in hypoxic ECs by Ang-1, possibly leading to Tie-2 receptor upregulation (556). However, the functional significance of STATs, Elk-1 and NERF2 in Ang-1/Tie-2 biological action is unknown.

The molecular mechanisms underlying the vascular effects of Ang-1 have just begun to be elucidated. It is not very clear yet whether Ang-1-induced effects are conveyed indirectly through the release of soluble mediators or through induction of insoluble factors. One of the first studies that tackled this issue demonstrated that Ang-1-induced recruitment of SMCs to ECs is mediated, in part, by the Ang-1-induced endothelial release of heparin binding EGF-like growth factor (HB-EGF). HB-EGF acts on the ERB1 and ERB2 receptors expressed on the surface of SMCs (557). A few years later, Kobayashi *et al.* showed that hepatocyte growth factor (HGF) is yet another factor released from ECs upon stimulation with Ang-1. This Ang-1-induced HGF is also involved in the vascular maturation process, as evidenced by enhanced migration of SMCs towards ECs, an effect that is antagonized by Ang-2 (558). Similarly, occludin, a transmembrane protein involved in the formation of tight junctions (TJ) between ECs of the blood brain barrier (BBB), has been found to be upregulated in brain capillary ECs following Tie-2 activation by multimeric angiopoietin-1 secreted by the adjacent brain pericytes (559). In addition, Ang-1 has been shown to induce expression of the anti-apoptotic molecule survivin and to reduce the cytosolic levels of the pro-apoptotic molecule Smac, thus leading to the protection of ECs from death induced by serum withdrawal (342;343). Finally, Ang-1 is able to induce the sprouting of ECs *in vitro* and neovascularization *in vivo* by enhancing the secretion of proteases such as plasmin, MMP-2 and MMP-9, and reducing the secretion of tissue inhibitors of metalloproteinase type 2 (TIMP2) (339;354;355).

1.9.1. Specific objectives of the thesis

Thus far, it has never been investigated whether these above-mentioned factors, or yet unknown factors, play a role in modulating Ang-1 effects on EC proliferation and migration. The **specific objective of this thesis** is, therefore, to identify the transcriptome of Ang-1 activated Tie-2 receptor in ECs and to further elucidate the signalling pathways, the transcription factors and the soluble mediators involved in the biological effects of Ang-1 in the endothelium.

To that end, we have used microarray technology to identify the genes that are regulated by Ang-1 in ECs (177). We were the first to show that interleukin-8 (CXCL8), a known pro-angiogenic chemokine, is induced by Ang-1 treatment. We further demonstrated that this Ang-1-induced CXCL8 plays an important autocrine role in Ang-1-induced EC proliferation and migration. In addition, we elucidated the mechanisms by which Ang-1 induces endothelial CXCL8 expression (358). Furthermore, our microarray data revealed induced expression of the transcription factor Egr-1 by Ang-1 treatment of ECs. Our investigations have demonstrated that Egr-1 upregulation following Ang-1 stimulation of ECs also plays a role in Ang-1-induced *in vitro* angiogenesis (manuscript submitted).

CHAPTER 2

TRANSCRIPTOME OF ANGIOPOIETIN 1-ACTIVATED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

N. A. Abdel-Malak, R. Harfouche and S. N.A. Hussain

Critical Care and Respiratory Divisions, Royal Victoria Hospital and Meakins-Christie Laboratories, McGill University Health Centre, McGill University, Montreal, Québec, Canada

Running Title: Angiopoietin/Tie-2 receptor signaling

Address of Correspondence:
Dr. S. Hussain,
Critical Care Division,
Royal Victoria Hospital,
Room L3.05,
687 Pine Ave West,
Montréal, Québec,
Canada H3A 1A1
Tel. 514 843 1664
Fax 514 843 1686

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2.1. ABSTRACT

Angiopoietin 1 (Ang-1) is the main ligand for endothelial cell (EC)-specific tyrosine kinase (Tie-2) receptors and it promotes migration and proliferation and inhibits apoptosis and vascular leakage. The exact mechanisms through which the Ang-1 exerts these effects remain unclear. We exposed human umbilical vein endothelial cells (HUVECs) to Ang-1 (300 ng/ml) for 4 h and conducted gene expression profiling using oligonucleotide microarrays. Real-time PCR was also conducted to verify several of the genes that were regulated by Ang-1. Exposure to Ang-1 resulted in induction of eighty-six genes that are involved in EC proliferation, differentiation, migration and survival. Thirty-six of these genes, including stanniocalcin, cyclin D1, vascular endothelial growth factor C, fms-related tyrosine kinase 1, interleukin 8 and CXCR4 have previously been shown to be induced by VEGF suggesting significant similarities between VEGF and Ang-1 pathways. Ang-1 exposure also inhibited mRNA expressions of 49 genes, most of which are involved in cell cycle arrest, apoptosis and suppression of transcription. These results indicate that Ang-1 triggers coordinated responses in ECs designed to inhibit the expression of pro-apoptotic and anti-proliferative genes and upregulate pro-proliferative, pro-angiogenic and anti-apoptotic pathways. Moreover, we also found that the Erk1/2, PI-3 kinase and the mTOR pathways are involved in Ang-1-induced gene expression in HUVECs.

Key Words: Angiogenesis, Tyrosine kinase receptors, Proliferation, Apoptosis, Angiopoietins

2.2. INTRODUCTION

Angiopoietin 1 (Ang-1 or ANGPT1) and its tyrosine kinase receptors (Tie-2 or TEK) have recently been identified as modulators of physiological and pathological angiogenesis. Genetic deletion of Ang-1 or Tie-2 in mice embryos is associated with major vascular defects and subsequent embryonic lethality, a result of loss of endothelial integrity (Sato *et al.*, 1995;Suri *et al.*, 1996). In cultured ECs, exposure to Ang-1 enhances sprouting, migration, and differentiation and induces a mild proliferative response (Kim *et al.*, 2000a;Witzenbichler *et al.*, 1998). Moreover, Ang-1 increases collateral vessel formation in ischemia-induced angiogenesis and exerts important anti-inflammatory responses, as evidenced by attenuation of leukocyte adhesion to ECs and reduction in the expression of adhesion molecules induced by tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) (Gamble *et al.*, 2000;Kim *et al.*, 2001;Shyu *et al.*, 1998).

The mechanisms through which the Ang-1/Tie-2 receptor system mediates its biological effects remain under investigation. Several reports have confirmed that activated Tie-2 receptors recruit adaptor proteins, including GRB2, GRB7, DOK-R and the p85 subunit of the PI3 kinase protein (PIK3R) (Jones *et al.*, 1999). Moreover, Tie-2 receptor phosphorylation is followed by activation of several pathways, including the PI3 kinase, protein kinase B (AKT1), p21 activated protein kinase (PAK1), focal adhesion kinase (FAK) pathways, as well as three members of the mitogen activated protein kinases (MAPKs), including ERK1/2 (also known as MAPK3/1), p38 and SAPK/JNK proteins, (Kim *et al.*, 2000a;Jones *et al.*, 1999;Master *et al.*, 2001;Harfouche *et al.*, 2003). Recent studies have revealed that inhibition of these pathways, particularly the AKT1 and the ERK1/2 pathways, results in attenuation of Ang-1-induced differentiation, sprouting, proliferation and survival of ECs (Harfouche *et al.*, 2003;Kanda *et al.*, 2005;Kim *et al.*, 2000b). Despite recent progress in elucidating the pathways that are activated by Ang-1 exposure in ECs, the downstream effectors through which these pathways mediate the biological effects of Ang-1 remain unclear. Only recently have investigators revealed that both heparin binding EGF-like growth factor (HBEGF) and hepatocyte growth factor (HGF) are released from ECs in response to Ang-1, and that these two growth factors

enhance smooth muscle cell (SMC) and pericyte recruitment towards ECs (Iivanainen *et al.*, 2003;Kobayashi *et al.*, 2006).

To delineate the mechanisms of action of Ang-1 in ECs, we measured alterations in the EC transcriptome by using oligonucleotide microarray technology. Our results indicate that exposure of ECs to Ang-1 triggers significant alterations in numerous genes involved in the regulation of cell cycle, proliferation, apoptosis, transcription, differentiation and inflammatory response. Overall responses appear to be designed to enhance EC proliferation, survival and differentiation and to inhibit apoptosis and inflammatory responses.

2.3. MATERIALS AND METHODS

Materials

HUVECs were purchased from GlycoTech Corporation (Gaithersburg, MD). Most cell culture reagents were obtained from Invitrogen Corporation (Burlington, ON). Endothelial cell growth supplement was supplied by Biomedical Technologies, Inc. (Stoughton, MA). Recombinant human Ang-1 protein and its cross-linking antibody were purchased from R&D Systems (Minneapolis, MN). The RNeasy Mini Kit for RNA isolation was supplied by Qiagen Inc. (Mississauga, ON).

Cell culture

HUVECs were cultured in endothelial basal medium MCDB131 supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement, 2mM glutamine and heparin. Prior to treatment with Ang-1, cells were washed then serum starved for 6 h with serum starvation medium, which consists of basal medium MCDB131 with 2mM glutamine and heparin. Cells were then treated either with Ang-1 vehicle (phosphate buffered saline (PBS), control) or 300 ng/ml of Ang-1 and collected 4 h later.

Microarray analysis

Cells were harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit, following the manufacturer's instructions. Quantification and purity of total RNA

was assessed by A_{260}/A_{280} absorption and by electrophoresis in 1.2 % agarose gel. Reverse transcription of 10 μg of RNA produced cRNA samples that were hybridized onto Affymetrix GeneChip arrays (HG-U133A) (Affymetrix, Inc., Santa Clara, CA), which contain 22,283 probe sets representing approximately 14,500 human genes. Probe labeling and hybridization were performed according to a standardized protocol supplied by Affymetrix. Hybridized arrays were run on the GeneChip Scanner 3000. GeneChip® Operating Software (GCOS) Version 1.0 was used to generate raw images (Cel files) and to subsequently reduce them to intensity values for each probe. Probe intensities were then processed using the robust multi-array analysis (RMA) algorithm. RMA is a log 2-scale linear additive model that summarizes perfect match probe values after correcting for background and quantile, thus normalizing probe level data across arrays (Irizarry *et al.*, 2003a;Bolstad *et al.*, 2003;Irizarry *et al.*, 2003b). RMA expression values were subsequently filtered using Microsoft Excel. Genes with a log 2 ratio greater than 0.59 or lower than -0.59 (±1.5 fold) were considered to be significantly regulated by Ang-1. To validate the results of the microarray experiments, we performed the following procedures:

A) Quantitative real-time PCR analysis:

Microarray data of eight upregulated and six downregulated genes were validated using real-time polymerase chain reaction (PCR) analysis of 4 independent experiments. For upregulated genes, we evaluated the expressions of two transcription factors (early growth response factor-1, EGR1, and kruppel-like factor 2, KLF2), the chemokine receptors CXCR4, plasminogen activator urokinase receptor (PLAUR), BCL2-related protein A1 (BCL2A1) and vascular endothelial growth factor C (VEGFC). All of these genes positively regulate EC proliferation, differentiation and survival (Feinberg *et al.*, 2004;Fahmy *et al.*, 2003;Oloffson *et al.*, 1998;Salcedo *et al.*, 1999;Gerber *et al.*, 1998). In addition, we measured the expression of stanniocalcin (STC1), a gene whose expression underwent the largest relative induction in its expression in response to Ang-1 exposure (see below). Finally, we also verified the expression of angiopoietin-like 4 (Anglk-4) that regulates EC proliferation and differentiation but has never been linked to the Ang-1/Tie-2 receptor pathway. For downregulated genes, we verified the expressions

of thrombospondin 1 (THBS1), growth arrest and DNA-damage-inducible beta (GADD45B), sulfatase 1 (SULF1), inhibitor of DNA binding 1 and 3 dominant negative helix-loop-helix proteins (ID1 and ID3) and angiopoietin 2 (Ang-2 or ANGPT2). All of these genes are involved in the regulation of cell differentiation, proliferation and survival (Lyden *et al.*, 1999;Narita *et al.*, 2006;Jimenez *et al.*, 2000;Wang *et al.*, 1999;Brindle *et al.*, 2006). For these experiments, serum starved HUVECs (in triplicates) were treated with PBS (control) or Ang-1 (300 ng/ml) for 1, 4, 8 and 24 h. Cells were collected and RNA was extracted, as described above. Two μg of RNA were reverse transcribed using the SuperScript II RNase H-Reverse Transcriptase enzyme from Invitrogen. Reactions were incubated at 42 °C for 50 min and at 90 °C for 5 min. Real-time PCR was performed using the Prism 7000 Sequence Detection System from Applied Biosystems (Foster City, CA) and specific primers (Table 1). We also monitored the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control gene. One μl of the reverse transcriptase reaction was added to 25 μl of SYBR Green PCR Master Mix (Qiagen Inc) and 3.5 μl of each 10μM primer. The thermal profile was as follows: 95 °C for 15 min, and 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72°C for 33 s. A melt analysis for each PCR experiment was used to assess primer-dimer formation or contamination. A single melt peak observed for each set of primers was used to validate that only a single PCR reaction was generated. Results were analyzed using the comparative threshold cycle (C_T, the value where the amplification curve crosses the threshold line) method where:

$$\Delta C_T = C_T \text{ of gene of interest} - C_T \text{ of GAPDH}$$

$$\Delta\Delta C_T = \Delta C_T \text{ of Ang-1 treated} - \Delta C_T \text{ of control}$$

Relative expression at a given time point was calculated as $2^{-\Delta\Delta CT}$. All real-time PCR experiments were performed in triplicate.

B) ELISA for IL-8 protein:

Serum starved HUVECs were treated with PBS (control) or Ang-1 (300 ng/ml) for 1, 6 and 12 h. The levels of secreted IL-8 in the culture supernatants were measured using a commercial IL8-ELISA kit from R&D Systems (Minneapolis, MN).

C) Expression of EGR1 protein and EGR1/DNA binding:

We verified EGR1 protein expression in response to Ang-1 exposure and detected nuclear mobilization of this protein by fractionation of cell lysates into cytosolic and nuclear fractions. Serum starved HUVECs were treated with PBS (control) or Ang-1 (300 ng/ml) for 1 h. Cells were harvested by scraping and were initially lysed with 400 µl of ice-cold lysis buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 5 µg/ml aprotinin, 1 µg/ml pepstatin, and 2 µg/ml leupeptin). After 15 min on ice, nonidet P-40 was added to a final concentration of 0.6% (v/v), and nuclei were centrifuged at 14000 rpm for 5 min. Supernatants containing cytoplasmic proteins were stored at -70 °C. The pelleted nuclei were re-suspended in 50 µl of ice-cold high salt buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 5 µg/ml aprotinin, 1 µg/ml pepstatin, and 2 µg/ml leupeptin). The lysed nuclei were vigorously rocked for 15 min at 4 °C and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants containing nuclear proteins were aliquoted and stored at -70 °C until used for immunoblotting (see below).

We also verified that Ang-1 exposure triggers an increase in EGR1 DNA binding by performing gel shift assays. Double-stranded oligonucleotides containing wild-type (WT) and mutant sequences (synthesized by Invitrogen) were annealed in 100 nM Tris-HCl (pH 7.5), 1 M NaCl, and 10 mM EDTA at 65 °C for 10 min; cooled for 1-2 h at room temperature; and finally end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Promega, Madison, WI). The sense sequences of the oligonucleotides tested were as follows: consensus WT Egr-1, 5'-GGA TCC AGC GGG GGC GAG CGG GGG CGA-3'; consensus mutant Egr-1, 5'-GGA TCC AGC GGG TAC GAG CGG GTA CGA-3'. Binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl2, 4% glycerol, and 2.5 µg of poly (dI-dC). Binding reactions were conducted with 15 µg of nuclear extract and 100,000 cpm ^{32}P-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 µl. For supershift analysis, 2 µg of affinity-purified anti-EGR1 polyclonal antibody was incubated for 10 min with the nuclear extracts prior to the addition of the radioactive probe. Binding complexes were resolved by non-denaturing polyacrylamide gel

electrophoresis on 6% gels in a tris borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

D) Activation of Erk1/2, PI-3 kinase and mTOR pathways:

We verified in these experiments activation of the Erk1/2, PI-3 kinase and the mammalian target of rapamycin (mTOR) pathways by Ang-1 in HUVECs. Serum starved HUVECs were exposed to PBS (control) or Ang-1 (300 ng/ml) for 15 min in the absence and presence of 1 h pre-incubation with PD98059 (30 μM, selective inhibitor of MEK1), wortmannin (100 nM, selective inhibitor of the PI-3 kinase pathway) and rapamycin (50 ng/ml, selective inhibitor of mTOR). Cells were harvested by scraping and were lysed with 400 μl of ice-cold lysis buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 2 μg/ml leupeptin). Total cell lysates underwent immunoblotting for total Erk1/2, phospho-Erk1/2 (Thr^{202}/Tyr^{204}), protein kinase B (AKT), phospho-AKT (Ser^{473}), total mTOR, phospho-mTOR (Ser^{2481}), total P70S6 and phospho-P70S6 kinase (Thr^{389}) (see below).

E) Differential gene regulation by the Erk1/2, PI-3 kinase and mTOR pathways:

To evaluate whether these three pathways differentially regulate gene expression in response to Ang-1, we exposed cells to Ang-1 (300 ng/ml) for 1 or 4 h in the presence of PD98059, wortmannin, and rapamycin and evaluated mRNA levels of three genes (EGR1, VEGFC, and ANGPTL4) using real-time PCR as described above.

Biological pathway analyses

Ingenuity Pathway Analysis system (Ingenuity Systems, www.ingenuity.com) was used to visualize gene expression data in the context of biological pathways. An RMA threshold of 1.5 was used to identify up- or downregulated genes. Three rounds of analyses were performed with the ingenuity Pathway Analysis system considering upregulated genes, downregulated genes and both up- and downregulated genes.

Immunoblotting

Total cell lysates, cytosolic and nuclear proteins (20 μg total protein) were boiled for 5 min and then loaded onto tris-glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked with 5% non-fat dry milk, and subsequently incubated with polyclonal antibodies for total Erk1/2, phospho-Erk1/2 ((Thr^{202}/Tyr^{204}), total AKT, phospho-AKT (Ser^{473}), m-TOR, phospho-mTOR (Ser^{2481}), P70S6 and phospho-P70S6 kinase (Thr^{389}). All of these antibodies were obtained from Cell Signaling Inc. (Danvers, MA). We also detected EGR1 protein in the cytosolic and nuclear fractions using a polyclonal anti-EGR1 (Santa Cruz Biotechnology, Santa Cruz CA). Equal protein loading was verified using anti-actin antibody (Santa Cruz Biotechnology). Proteins were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibodies and ECL reagents (Chemicon, Temecula, CA). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the Bio-Rad protein assay with BSA as a standard (Bio-Rad Laboratories, Canada, Mississauga, ON).

2.4. RESULTS

Microarray results

Upregulated genes

Figure 2.1 illustrates the differences in the distribution of log2 of gene expression intensities obtained from PBS-treated (control, x-axis) or Ang-1-treated (y-axis) HUVECs. The upregulated genes are shown as squares and the downregulated genes as triangles. The non-regulated genes are shown as dots. Eighty-six genes were significantly upregulated by Ang-1 (Table 2.2 and Figure 2.2). The largest relative increase in gene expression was detected in STC1 (4.6 fold), which is involved in the regulation of intracellular Ca^{++} concentration. Two regulators of apoptosis, namely, BCL2A1 (an anti-apoptotic member of the BCL2 family) and CARD8 (of the caspase recruitment domain family) were also significantly upregulated by Ang-1. Many proteins involved in EC proliferation and migration including VEGFC, VEGF receptor 1 (FLT1), platelet derived

growth factor A (PDGFA), PLAUR, interleukin 8 (IL8), CXCR4, and cyclin D1 (CCND1) were significantly enhanced by Ang-1 (Table 2.2). In addition, six transcription factors were induced by Ang-1, including early growth response 1 (EGR1) and Kruppel-like factor 2 (KLF2). Figure 2.3 illustrates the time course of mRNA expression of EGR1, BCL2A1, ANGPTL4, CXCR4, PLAUR, STC1, VEGFC, and KLF2 in response to 1, 4, 8 and 24 h exposures to Ang-1 (300 ng/ml). The expression of each of these genes was significantly induced in response to Ang-1 albeit to different extents and according to different time courses. Whereas EGR1, BCL2A1, and KLF2 showed early induction (within 1 h) with a subsequent decline to control levels thereafter, the expression of ANGPTL4, CXCR4, PLAUR, STC1, and VEGFC rose after 4 or 8 h of Ang-1 exposure (Figure 2.3). Figure 2.4A shows that Ang-1 exposure elicited after 6 and 12 h of exposure a significant rise in IL8 protein levels compared with PBS (control). In addition to measuring EGR1 mRNA expression, we documented that Ang-1 exposure triggers significant induction of EGR1 protein by immunoblotting with anti-EGR1 antibody of cytosolic and nuclear lysates of HUVECs after 1 h exposure to PBS (control) or Ang-1 (Figure 2.4B). Moreover, gel shift assays using oligonucleotides corresponding to EGR1 consensus binding sequence revealed that Ang-1 significantly induced EGR1 DNA binding (Figure 2.4C). Supershift assays using anti-EGR1 antibody impaired the formation of EGR1/oligonucleotide complex. The specificity of EGR1/DNA binding was also confirmed by elimination of this binding when the labeled oligonucleotides were competing with cold wild type oligonucleotides whereas competition with mutated oligonucleotides had no effect on EGR1/DNA binding (Figure 2.4C).

Signaling pathways activated by Ang-1

Figure 2.5 shows the influence of Ang-1 on phosphorylation of Erk1/2, AKT, mTOR and P70S6 kinase. Ang-1 exposure for 15 min elicited a significant increase in Erk1/2 phosphorylation, which was attenuated by the MEK-1 inhibitor, PD98059. Similarly, AKT phosphorylation on Ser^{473} was significantly increased in response to Ang-1, a response that was attenuated by pre-incubation with a selective PI-3 kinase inhibitor (wortmannin, WM) (Figure 2.5B). Ang-1 also triggered an increase in mTOR phosphorylation on Ser^{2481} and P70S6 kinase phosphorylation on Thr^{389} (Figure 2.5C and

D). Ang-1-induced P70S6 kinase phosphorylation was attenuated by both rapamycin and wortmannin suggesting that Ang-1/Tie-2 receptor pathway activates the P70S6 kinase through the mTOR and the PI-3 kinase pathways (Figure 2.5D). To assess whether the Erk1/2, PI-3 kinase and mTOR pathways selectively regulate gene expression downstream from Tie-2 receptors, we selected EGR1, ANGPTL4 and VEGFC which are included into the top three networks of upregulated genes (see below) and measured their mRNA expressions after 1 and 4 h of Ang-1 exposure in the absence and presence of PD98059, wortmannin and rapamycin. These three inhibitors significantly attenuated Ang-1-induced EGR1 expression suggesting that Erk1/2, PI-3 kinase and mTOR pathways are all involved in its induction in the presence of Ang-1. By comparison, wortmannin and rapamycin but not PD98059 reduced ANGPTL4 mRNA expression triggered by Ang-1 thereby implicating the PI-3 kinase and mTOR in the regulation of this gene during Ang-1 exposure (Figure 2.6). With respect to VEGFC, only wortmannin had an effect on its regulation in the presence of Ang-1 thereby excluding the Erk1/2 and mTOR pathways in this response (Figure 2.6).

Downregulated genes

Table 2.3 lists the genes that were significantly downregulated by Ang-1. A total of forty-nine genes were attenuated by Ang-1, with chromosome 8 open reading frame 4 (C8orf4, also known as TC1) being the most strongly inhibited gene (Table 2.3). Little is known about the function of this gene, which was initially cloned in thyroid cancer samples (hence, the name TC1). Many pro-inflammatory, pro-apoptotic and anti-proliferative genes were also downregulated by Ang-1 (Figure 2.2), including tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10, TRAIL), bone morphogenetic protein 4 (BMP4), growth arrest and DNA-damage-inducible factor, beta (GADD45B), thrombospondin 1 (THBS1) and sulfatase 1(SULF1) (Table 2.3). Moreover, three members of the inhibitor of DNA binding dominant negative helix-loop-helix protein family (ID1, ID2 and ID3) and two inhibitors of transforming growth factor beta (TGFβ) receptor signaling- BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) (BAMBI) and Dachshund homolog 1 (Drosophila) (DACH1) - were significantly downregulated by Ang-1 (Table 2.3).

Figure 2.7 illustrates the time course of mRNA expression of six genes (THBS1, GADD45B, SULF1, ID1, ID3 and ANG2) in response to 1, 4, 8 and 24 h exposures to Ang-1 (300 ng/ml). THBS1 and SULF1 were downregulated within 1 h of Ang-1 exposure, whereas attenuation of GADD45B, ID1, ID3 and ANG2 was detectable at the 4 and 8 h marks (Figure 2.7).

Pathway analyses

The ingenuity Pathway Analysis software was used to analyze the microarray dataset in the context of biological pathways. Three runs of analyses were performed considering only upregulated genes, only downregulated genes and up- and downregulated genes. The top functions associated with the most significant network of upregulated genes were cancer, cellular growth and proliferation. Five major networks of upregulated genes were detected which contained 20, 18, 14, 11, 11 focus genes, respectively. The top network of upregulated genes is shown in Figure 2.8 and contains several highly connected nodes such as EGR1 and two components of the activator protein-1 complex, namely, c-Jun and Fra1 (FOSL1). Other highly connected nodes in this network include cyclin-D1 (CCND1) and prostglandin synthase 2 (PTGS2). These nodes interact directly and indirectly with several pro-angiogenesis genes including PDGF-A, PDGF-B, FLT1 receptors and PLAUR and are also modulated by the Erk1/2 pathway (Figure 2.8). The second top network of upregulated genes (data not shown) identified IL8, CXCR4, VEGF and KLF2 as the most highly connected nodes that interact with each other and are modulated by the PI-3 kinase/AKT, Erk1/2, p38 and SAPK/JNK pathways. Three main networks of downregulated genes were detected that contained 15, 13 and 8 focus genes (data not shown). The top functions associated with these networks were cancer, cellular growth and proliferation, gene expression, cell cycle, DNA replication and repair. The most highly connected nodes in the top network of downregulated genes include ID1, ID2 and ID3 transcription factors, THBS1, BMP4 and GADD45B. When both up- and downregulated genes were analyzed using the Ingenuity system, 8 main networks were detected with top functions including cancer, cellular growth and proliferation, cellular development and cell movement and signaling

(data not shown). These networks contained 23, 20, 17, 14, 12, 12, 11 and 6 focus genes, respectively.

2.5. DISCUSSION

Little is known about the genes that regulate the angiopoietin/tyrosine kinase (Ang-1/Tie-2) receptor system in ECs, despite their indispensability to the processes of vascular formation and angiogenesis. We sought to identify these genes by comparing the transcriptome of HUVECs exposed for 4h to Ang-1 to those exposed to PBS. In our study, eighty-six genes were selectively upregulated and forty-nine genes were significantly downregulated in response to Ang-1 exposure. Chen *et al.* (Chen *et al.*, 2005) have recently used serial analysis of gene expression (SAGE) to identify the underlying genetic mechanisms that regulate the transcriptome during vascular remodeling in the yolk sacs of day 8.5 Tie-$2^{+/+}$ and Tie-$2^{-/-}$ mice embryos. They reported that 14% of the entire tag population in these yolk sacs was differentially expressed by a more than two-fold factor. When one compares our results to those of Chen *et al.*, very little overlap in the expression of genes regulated by Tie-2 receptors is evident. We believe that the discrepancies between our results and theirs are due to several factors, including the methods used (SAGE *vs.* microarrays), but also as a result of species-specific biological factors inherent in the use of developing yolk sacs in mice versus the use of mature ECs exposed to exogenous Ang-1.

Aplin *et al.* (Aplin *et al.*, 2006) have recently reported that Ang-1 upregulates several innate immunity-related cytokines such as IL-1α, IL-β, MCP-1, GRO-1, MIP-1α, MIP-1β, MIP-2, MIP-3α and TNF-α in aortic rat rings. It is interesting to note that in our study the only chemokine that was significantly upregulated by Ang-1 was IL-8 (CXCL8). We speculate that the dissimilarity of our results with those of Aplin *et al.* can be attributed to methodological differences. For instance, we measured acute (4h) changes in the cellular transcriptome in homogenous cell population (HUVECs), whereas Aplin *et al* studied more prolonged (18h) responses elicited by Ang-1 in aortic rings, which contain many cell types, such as SMCs, ECs and adventitial macrophages. It is possible that the cytokines related to innate immunity that were upregulated by Ang-1 in the Aplin *et al.* study might have been derived from adventitial macrophages and mural

cells. This speculation is based on the fact that Tie-2 receptors were detected in subpopulations of monocytes and periendothelial mural cells (Iurlaro *et al.*, 2003;De *et al.*, 2005).

Our current results indicate that, in HUVECs, Ang-1 regulates a program of gene expression that serves to promote proliferation, survival, differentiation and migration. To further analyze the Ang-1-induced program of gene expression in HUVECs, we used the Ingenuity Pathway Analysis System, which generated knowledge-based networks of regulated genes. A closer look at the most significant network of Ang-1-upregulated genes (Figure 2.8) reveals several highly connected nodes, such as EGR1, c-JUN, Fra1 and cyclin D1, that play major roles in the regulation of EC proliferation and migration. EGR1 is an immediate-early transcription factor that is highly induced by a variety of stimuli, including pro-angiogenic growth factors such as fibroblast growth factors (FGFs) and VEGF, fluid shear stress, lipopolysaccharides, thrombin, angiotensin II and hypoxia (for review, see (Khachigian, 2006)). EGR1 works in concert with other transcription factors including p65 NFκB, nuclear factor of activated T-cells (NFAT) and specificity protein 1 (SP1) (Khachigian, 2006). It also plays an important role in angiogenesis, since inhibition of EGR1 expression results in attenuation of microvascular EC proliferation, migration and microtubule network formation *in vitro*, inhibition of VEGF-inducible corneal neovascularization in rats, and reduction in tumor growth and tumor angiogenesis in mice (Fahmy *et al.*, 2003).

The network shown in Figure 2.8 predicts that various levels of the Erk1/2 pathway (Ras, Mek1/2 and Erk1/2 proteins) might regulate EGR1 expression. We confirmed that Ang-1 triggers upregulation of EGR1 expression in HUVECs, in part through the Erk1/2 pathway. Observations that EGR1 promotes EC proliferation and migration in response to a variety of growth factors and that it is highly induced in response to Ang-1 exposure in HUVECs suggest that this transcription factor may play important roles in the biological effects of Ang-1 on EC differentiation and sprouting.

Other highly connected nodes shown in Figure 2.8 are two subunits of the transcription factor activator protein-1 (AP-1), namely, c-Jun and Fra1. The AP-1 complex consists of Jun family homodimers (c-Jun, JunB and JunD), and Jun/Fos (c-Fos, FosB, Fra1 and Fra2) or Jun/ATF2 heterodimers (Shaulian and Karin, 2001). AP-1 is

activated and induced by a plethora of physiological and pathological stimuli and is involved in several cellular responses such as proliferation, migration and apoptosis (Shaulian and Karin, 2001). We have recently documented that Ang-1 triggers significant increases in AP-1 DNA binding and that this response is mediated through increased phosphorylation of c-Jun on Ser^{63} and Ser^{73} (Abdel-Malak *et al.*, 2007). In addition, we found that increased AP-1 DNA binding in response to Ang-1 exposure is mediated through the PI-3 kinase/AKT, SAPK/JNK and Erk1/2 pathways (Abdel-Malak *et al.*, 2007). These results indicate that in HUVECs, Ang-1 triggers a significant increase in DNA binding activity of not only EGR1, but that of the AP-1 complex as well.

The second most important network of upregulated genes in response to Ang-1 exposure indicates the chemokine IL8, the chemokine receptor CXCR4, the growth factors VEGFC and the transcription factor KLF2 as highly connected nodes. The fact that IL8, CXCR4 and VEGFC are induced in ECs undergoing differentiation into tube-like structures and in ECs exposed to VEGF (Bell *et al.*, 2001;Abe and Sato, 2001;Kahn *et al.*, 2000;Gerritsen *et al.*, 2003;Yang *et al.*, 2002) suggest significant similarities in the responses induced by Ang-1 and those elicited by other EC-specific growth factors. Although the exact roles played by these genes in biological responses to Ang-1 remain to be investigated, our own recent study reveals that Ang-1 triggers a significant induction of IL-8 in HUVECs and that this chemokine, acting in an autocrine fashion, promotes proliferation and migration of these cells (Abdel-Malak *et al.*, 2007).

Whereas there are similarities in the transcriptome changes elicited by Ang-1 in HUVECs to those elicited by other growth factors, there are several genes that were uniquely induced by Ang-1. One such example is the transcription factor KLF2, a member of the zinc finger protein family that is involved in the regulation of several cellular processes, including cellular growth and differentiation (Feinberg *et al.*, 2004). KLF2 is strongly induced in ECs by fluid shear stress and has strong anti-inflammatory effects (SenBanerjee *et al.*, 2004). Another clear disparity in EC transcriptome alterations triggered by Ang-1, as compared to other angiogenesis factors, is related to ANG2 (Ang-2) expression, which is induced in ECs exposed to VEGF or ECs undergoing differentiation into tube-like structures (Bell *et al.*, 2001;Abe and Sato, 2001;Kahn *et al.*, 2000;Gerritsen *et al.*, 2003;Yang *et al.*, 2002). In our study, however, Ang-1 elicited a

significant inhibition of EC Ang-2. This finding is in agreement with results that have demonstrated the downregulation of Ang-2 mRNA by Ang-1 in bovine microvascular ECs (Mandriota and Pepper, 1998). The biological significance of this observation remains speculative, although several investigators have proposed that the role of Ang-2 in angiogenesis is context-dependent. By antagonizing the stabilizing effect of Ang-1 on the vasculature, Ang-2 might function as a permissive angiogenic signal in the presence of VEGF and other angiogenic factors, thereby promoting EC migration and proliferation during early phases of angiogenesis (Mandriota and Pepper, 1998;Holash *et al.*, 1999). However, during late stages of angiogenesis, where correct vascular assembly and recruitment of perivascular cells is necessary, an increase in Ang-1 production, coupled with attenuation of Ang-2 release, is required for proper activation of Tie-2 receptors (Mandriota and Pepper, 1998;Holash *et al.*, 1999).

With respect to the inhibitory influence of Ang-1 on gene expression, the Ingenuity Pathway Analysis system reveals that the top network of downregulated genes contains several highly connected nodes, including ID1, ID2 and ID3 transcription factors, THBS1, BMP4 and GADD45B. The ID transcription factors belong to the inhibitor of DNA binding (ID) family, which are transcriptional regulators that contain a helix-loop-helix domain (HLH). ID proteins inhibit the functions of basic helix-loop-helix transcription factors in a dominant-negative manner by suppressing their heterodimerization partners through the HLH domains (Kadesch, 1993). Many reports have confirmed that overexpression of ID1 protein in various cells, including ECs, leads to a delay in senescence; however, the roles of ID proteins in angiogenesis remain unclear. The fact that ID proteins are negative regulators of HLH transcription factors, which are critical for the expressions of genes associated with cellular differentiation, suggests that inhibition of ID protein expression might promote EC differentiation. Indeed, Bell *et al.* (2001) have reported that the expression of ID1, ID2 and ID3 in ECs declines significantly during capillary morphogenesis in 3D collagen matrices.

There is increasing evidence that the PI-3 kinase/AKT, Erk1/2, p38 and SAPK/JNK pathways are activated downstream from Tie-2 receptors in ECs (Harfouche *et al.*, 2003;Harfouche *et al.*, 2005;Papapetropoulos *et al.*, 2000;Kim *et al.*, 2000b). These pathways play central roles in regulating the expression of many genes indicated in the

top networks of up- and downregulated genes generated by the Ingenuity Pathway Analysis System. The direction and the degree of gene expression regulation by these pathways differ significantly among various Ang-1-induced genes. For instance, whereas both the PI-3 kinase and Erk1/2 pathways contribute to the induction of EGR1 expression, the p38 pathway actually inhibits Ang-1-induced IL8 production in ECs (Abdel-Malak *et al.*, 2007).

In addition to the above-described pathways, we found that Ang-1 also activates mTOR, a serine/threonine protein kinase, which plays key roles in cellular growth, ribosome biogenesis, glucose and fat metabolism, autophagy, transcription and actin organization (Wullschleger *et al.*, 2006). The mTOR network regulates protein translation through P70S6 kinase and elongation factor 4E binding protein 1 (elF4E-BP1). Phosphorylation of 4E-BP1 releases the inhibitory effect of this protein on elF4E, which then triggers protein translation, whereas activated P70S6 protein phosphorylates the ribosomal protein S6, resulting in increased translation. We confirm here that P70S6 kinase is activated downstream from Tie-2 receptors as a result of activation of the mTOR network and the PI-3 kinase pathway since both rapamycin (an mTOR inhibitor) and wortmannin (a PI-3 kinase inhibitor) attenuated Ang-1-induced P70S6 phosphorylation. In addition, our results reveal that the mTOR network may participate in Ang-1-induced alterations in the regulation of expression of a few of the genes listed in Tables 1 and 2, however, the full extent of this participation needs further evaluation.

2.6. ACKNOWLEDGMENTS

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Figure 2.1: Differences in the distribution of log2 of gene expression intensities obtained from control (x-axis) and Ang-1 treated (y-axis) HUVECs.

Upregulated genes are shown as squares and downregulated genes as triangles. The non-regulated genes are shown as dots.

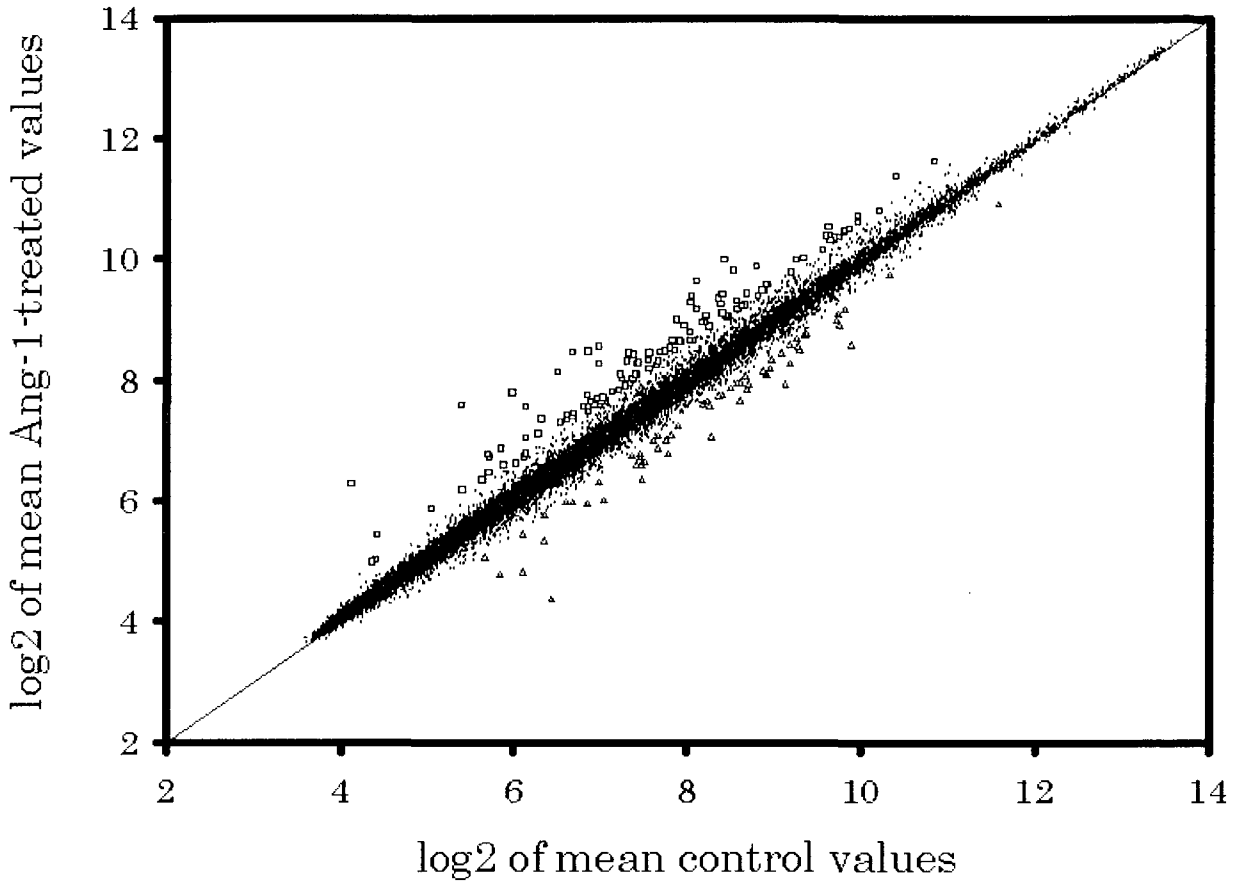
log2 of mean Ang-1-treated values
14
12
10
8
6
4
2
2
4
6
8
10
12
14
log2 of mean control values

FIGURE 2.1

Figure 2.2: Classification of genes that were upregulated and downregulated by Ang-1 exposure.

The Y-axis indicates the number of genes regulated by Ang-1 in each category.

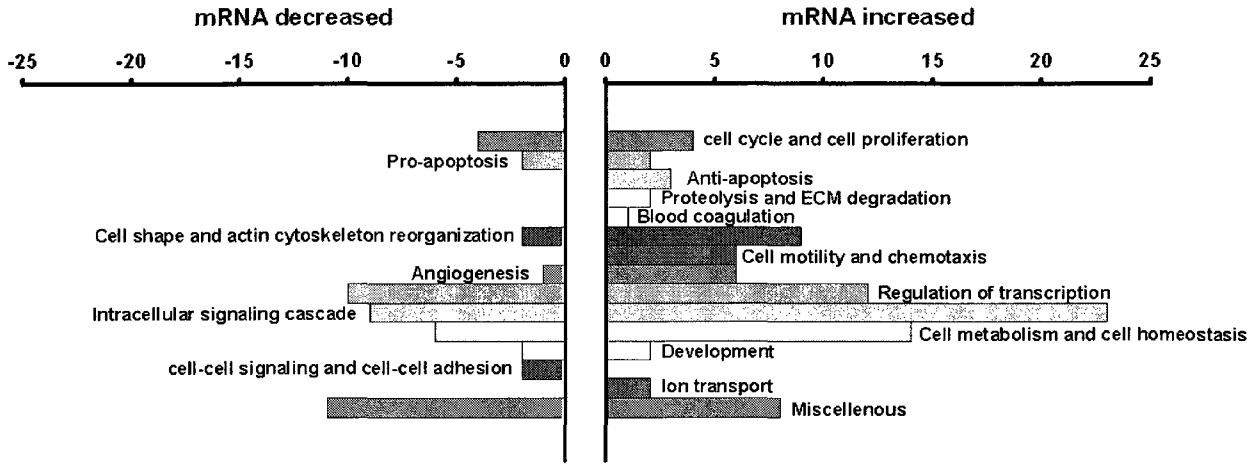
mRNA decreased
mRNA increased
-25
-20
-15
-10
-5
0
0
5
10
15
20
25
Pro-apoptosis
Cell shape and actin cytoskeleton reorganization
Angiogenesis
Intracellular signaling cascade
cell-cell signaling and cell-cell adhesion
cell cycle and cell proliferation
Anti-apoptosis
Proteolysis and ECM degradation
Blood coagulation
Cell motility and chemotaxis
Regulation of transcription
Cell metabolism and cell homeostasis
Development
Ion transport
Miscellenous

FIGURE 2.2

Figure 2.3: Ang-1-induced changes in mRNA of eight genes measured with Real-time PCR.

Results are expressed in relation to control values. HUVECs were exposed to PBS (control) or Ang-1 (300 ng/ml) and collected 1, 4, 8 and 24 h later. At each time point, mRNA expression in response to Ang-1 was measured and normalized as fold changes from the control samples. The interrupted lines indicate the levels of control values. Note the early induction of EGR1, BCL2A1 and KLF2 genes, whereas the other genes were induced after 4 or 8 h of Ang-1 exposure. * $P<0.05$ compared with control.

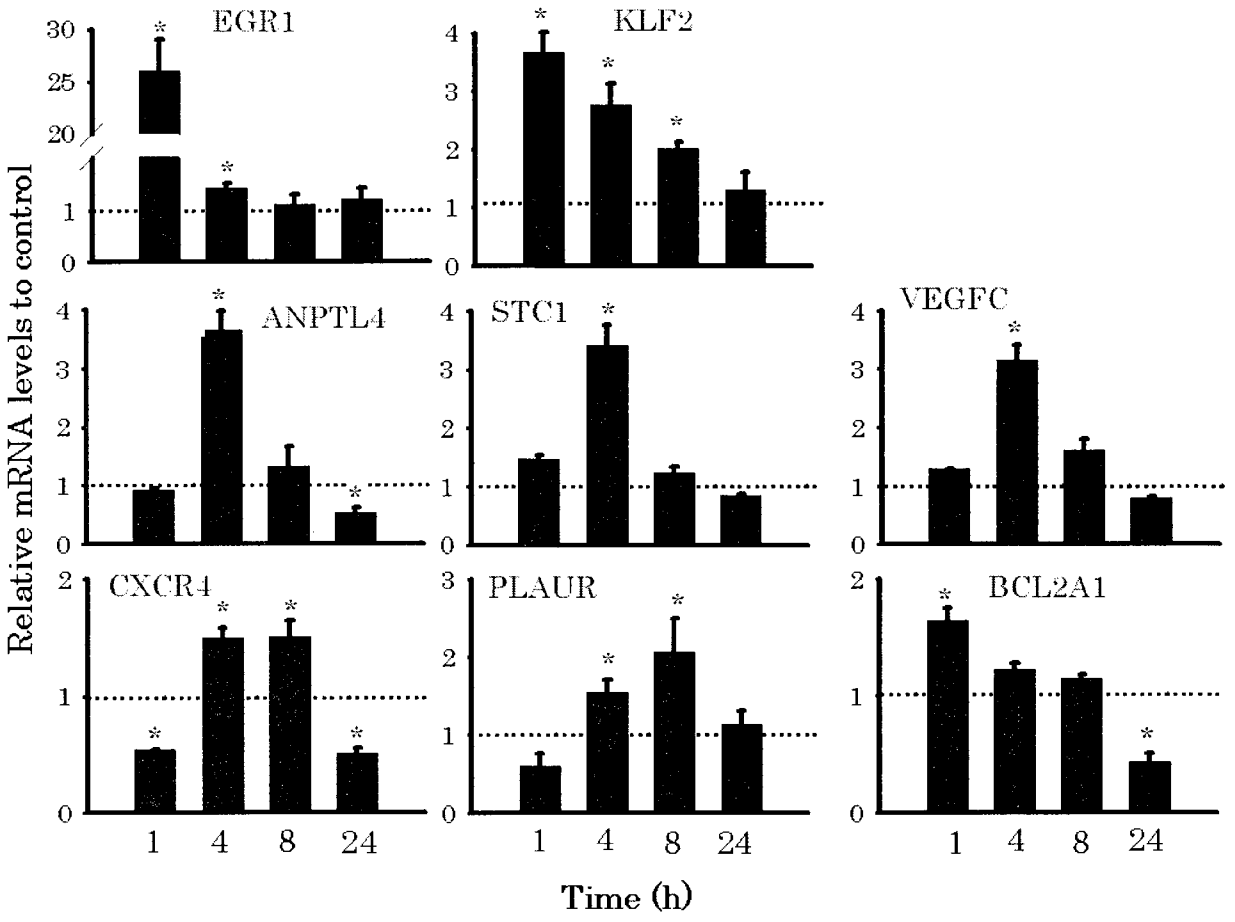
EGR1
KLF2
ANPTL4
STC1
VEGFC
CXCR4
PLAUR
BCL2A1
Relative mRNA levels to control
Time (h)
1
4
8
24

FIGURE 2.3

Figure 2.4: Measurements of IL-8 protein levels and Egr-1 protein expression and nuclear binding following Ang-1 treatment of HUVECs.

A: Measurement of IL-8 protein levels in the culture medium of HUVECs after 1, 6 and 12 h of PBS (control) or Ang-1 (300 ng/ml) treatment. Values are means±SEM of six independent observations in each group. *P<0.05 compared with control.

B: Expression of EGR1 protein in the nuclear (N) and cytosolic (C) fractions of total cell lysates after 1 h of treatment with PBS (control) or Ang-1 (300 ng/ml). Note the significant induction of EGR1 protein in the nuclear fraction in response to Ang-1 treatment.

C: Binding of nuclear extracts from control and Ang-1-treated HUVECs (1 h at 300 ng/ml) to specific EGR1 DNA oligonucleotides in the absence (-) and presence (+) of anti-EGR1 antibody. Competition with cold wild type and mutated oligonucleotides was performed as specificity control. SS: Supershifting.

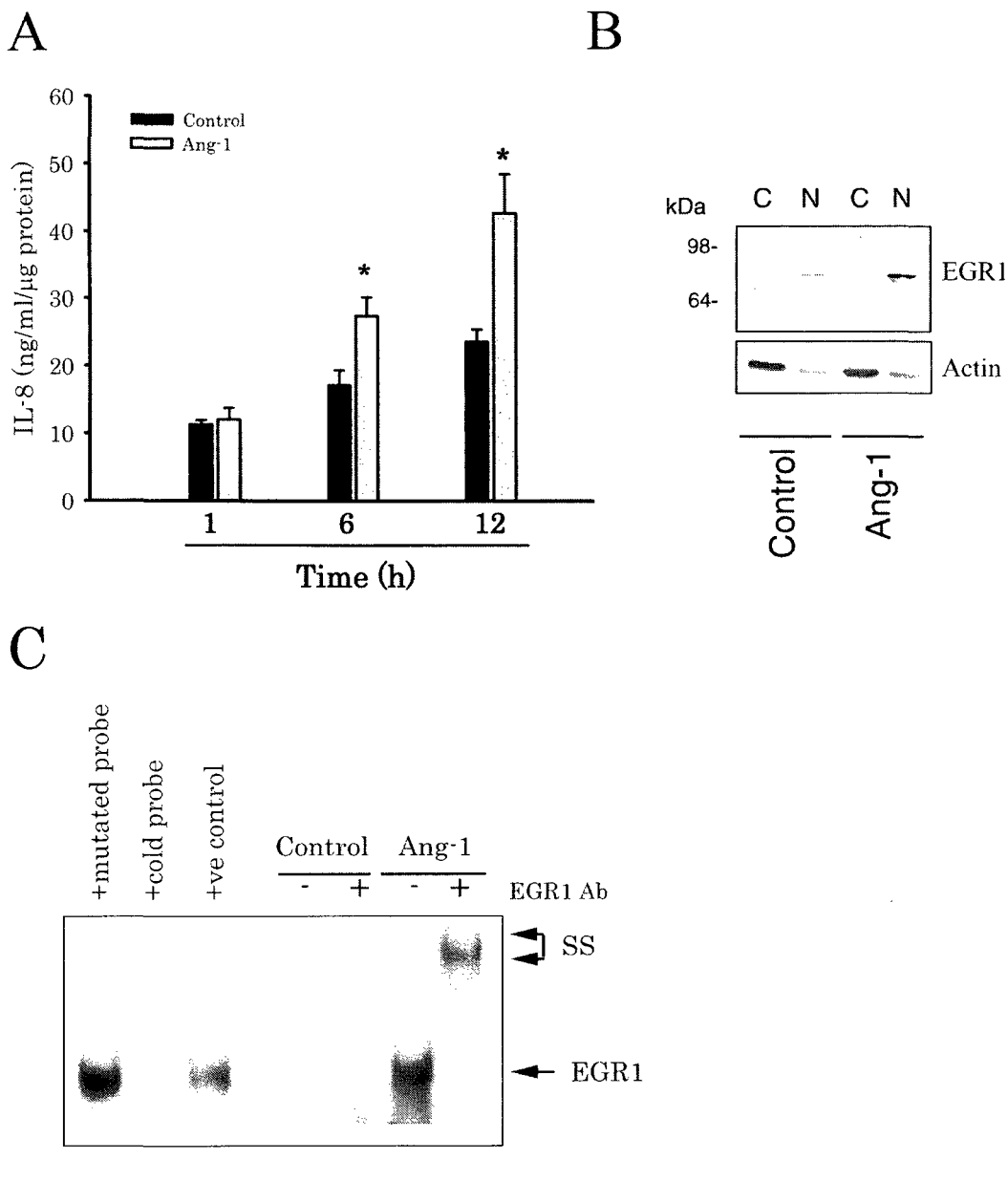
A
B
C
60
50
40
30
20
10
0
IL-8 (ng/ml/μg protein)
Control
Ang-1
*
*
1
6
12
Time (h)
kDa
C N C N
98-
64-
EGR1
Actin
Control
Ang-1
+mutated probe
+cold probe
+ve control
Control
Ang-1
- + - +
EGR1 Ab
SS
EGR1

FIGURE 2.4

Figure 2.5: Changes in Erk1/2, AKT, mTOR and P70S6 kinase phosphorylation after Ang-1 exposure.

Changes in Erk1/2, protein kinase B (AKT), mTOR and P70S6 kinase phosphorylation measured after 15 min (panels A, B, and D) and 120 min (panel C) of Ang-1 (300 ng/ml) exposure. Also shown are the effects of 1 h pre-incubation with PD98059, wortmannin (WM) and rapamycin on Erk1/2, AKT, and P70S60 kinase phosphorylation, respectively.

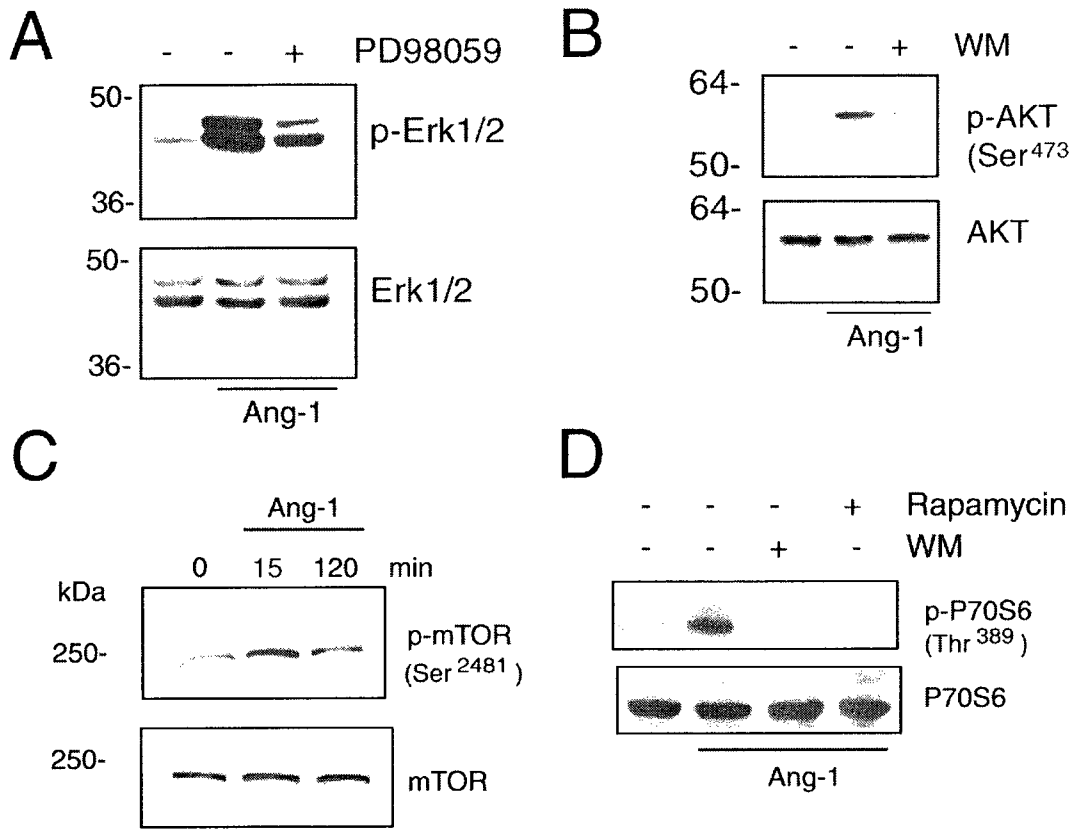
A
- - + PD98059
50-
36-
p-Erk1/2
50-
36-
Erk1/2
Ang-1
B
- - + WM
64-
50-
p-AKT
(Ser473)
64-
50-
AKT
Ang-1
C
Ang-1
0 15 120 min
kDa
250-
p-mTOR
(Ser2481)
250-
mTOR
D
- - - + Rapamycin
- - + - WM
p-P70S6
(Thr389)
P70S6
Ang-1

FIGURE 2.5

Figure 2.6: Influence of PD98059, wortmannin (WM) and rapamycin on Ang-1-induced augmentation of EGR1, ANGPTL4 and VEGFC mRNA expression.

The levels of mRNA were measured with Real-time PCR after 1 and 4 h of Ang-1 treatment in the absence and presence of PD98059, WM and rapamycin. Results are means and SEM of four independent measurements and are expressed as fold changes from those measured with Ang-1 alone. *$P<0.05$ compared with those measured with Ang-1 alone.

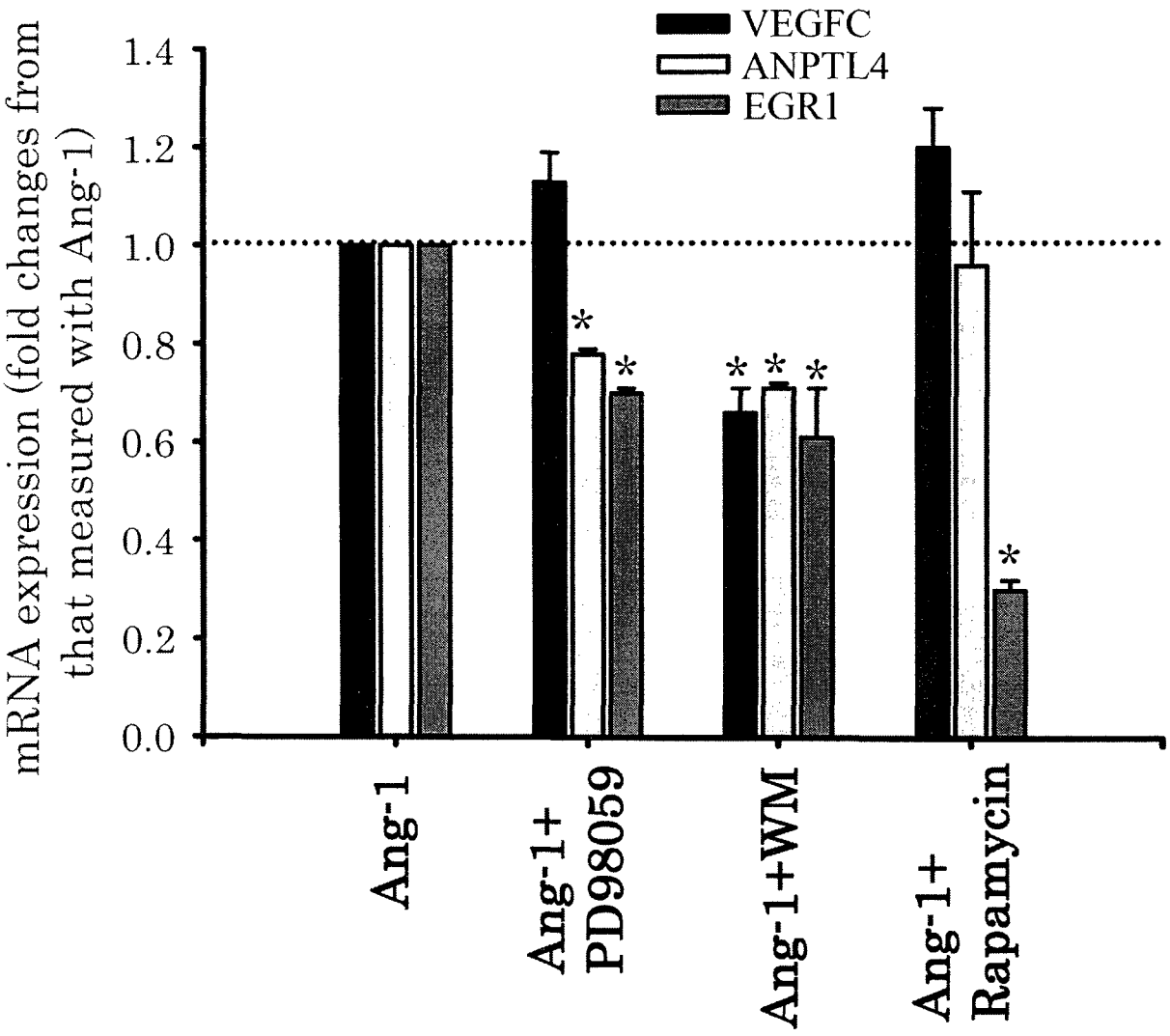
VEGFC
ANPTL4
EGR1
mRNA expression (fold changes from that measured with Ang-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
*
*
Ang-1
Ang-1+ PD98059
Ang-1+WM
Ang-1+ Rapamycin

FIGURE 2.6

Figure 2.7: Ang-1-induced down-regulation of six genes measured with Real-time PCR.

Results are expressed in relation to control values. Note the early downregulation of THBS1 and SULF1 whereas the expressions of ID1, ID2, ANG2 and GADD45 declined 4, 8 and 24 h after Ang-1 addition. *P<0.05 compared with control.

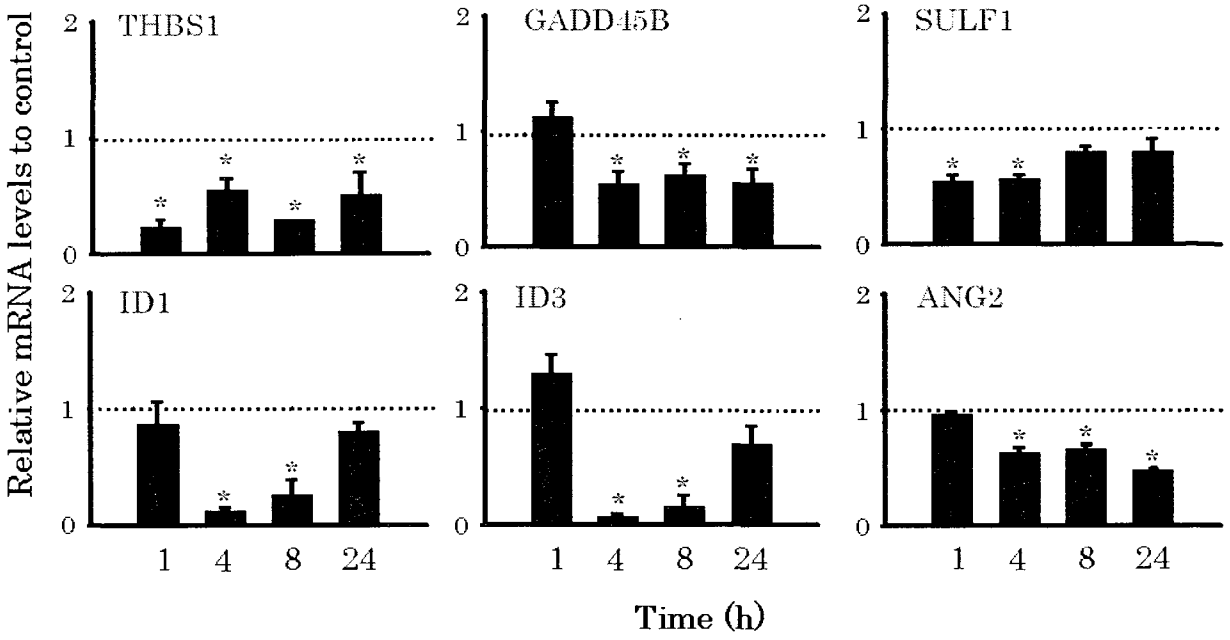
Relative mRNA levels to control
THBS1
GADD45B
SULF1
ID1
ID3
ANG2
2
1
0
1 4 8 24
Time (h)

FIGURE 2.7

Figure 2.8: The top network of upregulated genes in HUVECs exposed for 4 h to Ang-1.

This figure was created with the Ingenuity Pathway Analysis system. The microarray dataset was used as an input file with an RMA threshold of 1.5 to specify upregulated genes. Gene upregulated are shown in red. White nodes are gene absent from the microarray.

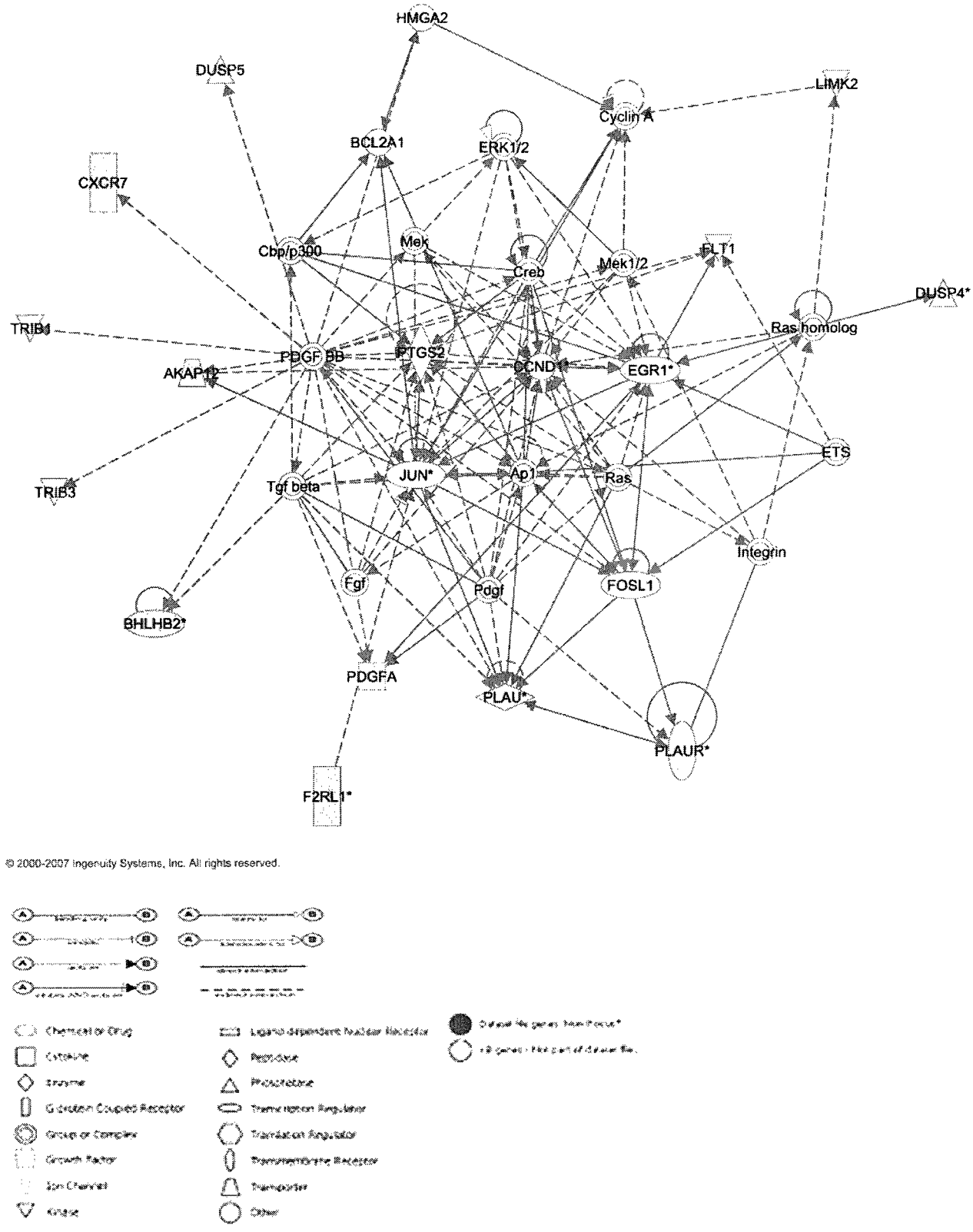
HMGA2
DUSP5
LIMK2
Cyclin A
BCL2A1
ERK1/2
CXCR7
Cbp/p300
Mek
Creb
Mek1/2
FLT1
DUSP4*
TRIB1
Ras homolog
PDGF BB
PTGS2
CCND1*
EGR1*
AKAP12
ETS
TRIB3
Tgf beta
JUN*
Ap1
Ras
Integrin
Fgf
Pdgf
FOSL1
BHLHB2*
PDGFA
PLAU*
PLAUR*
F2RL1*
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FIGURE 2.8

Table 2.1: Primers used for Real-Time PCR experiments.

Gene	Fwd Primer (Tm)	Reverse Primer (Tm)	Product size(bp)
ANPTL4	CCACTTGGGACCAGGATCAC	CGGAAGTACTGGCCGTTGAG	115
CXCR4	AGCATGACGGACAAGTACAGG	GATGAAGTCGGGAATAGTCAGC	309
PLAUR	CAGGAAGGTGAAGAAGGGCGT	TTGCAGCATTTCAGGAAGTGG	125
STC1	TGGTGATCAGTGCTTCTGCAAC	CTCAGTGATGGCTTCAGGGTTC	410
VEGFC	TGTACAAGTGTCAGCTAAGG	CCACATCTATACACACCTCC	183
KLF2	GCACGCACACAGGTGAGAAG	ACCAGTCACAGTTTGGGAGGG	269
THBS1	GACCCACGACTGCAACAAGAA	GTCTCCCACATCATCTCTGTCA	358
SULF1	CCACCTACCACTGTCCGAGT	TCTGCCGTCTCTTCTCCTTC	379
ID1	TGAGCTTGCTGGACGACATG	GATGACGCGCTGTAGGATTTC	110
ID3	GGCTGTTACTCACGCCTCAAG	CAAACTGAAGGTCCCTGATGTAG	112
Ang-2	ATAAGCAGCATCAGCCAACCA	CATTCCGTTCAAGTTGGAAGGA	136
GAPDH	AAGAAGGTGGTGAAGCAGGCG	ACCAGGAAATGAGCTTGACAA	166

Table 2.2: Genes that were upregulated by Ang-1 exposure.

Gene (Symbol)	Molecular Function(s)	Genbank ID	Fold induction
Cell cycle and cell proliferation:			
Epithelial membrane protein 1 (EMP1)	Adhesion molecule	NM_001423	1.9 *
Platelet-derived growth factor alpha polypeptide (PDGFA)	growth factor activity	NM_002607	1.8 *
Cyclin D1 (CCND1)	Regulation of cell cycle	NM_053056	1.7 *
KIT ligand (KITLG)	Growth factor activity	NM_000899	1.6 *
Anti-apoptosis:			
BCL2-related protein A1 (BCL2A1)	Apoptosis inhibitor activity	NM_004049	4.5 *
Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2)	Plasminogen activator activity (anti-apoptotic)	NM_002575	2.9 *
Pleckstrin homology-like domain, family A, member 2 (PHLDA2)	DNA binding (anti-apoptotic)	NM_003311	1.5 *
Pro-apoptosis:			
Caspase recruitment domain family, member 8 (CARD8)	Caspase activator activity	NM_014959	1.6
Tumor necrosis factor (ligand) superfamily,member 15 (TNFSF15)	Tumor necrosis factor receptor binding	NM_005118	1.6 *
Proteolysis and ECM degradation:			
Plasminogen activator, urokinase receptor (PLAUR)	Receptor activity	NM_002659	2.5 *
Plasminogen activator, urokinase (PLAU)	u-plasminogen activator activity	NM_002658	1.6 *

Blood coagulation:			
Plasminogen activator, urokinase receptor (PLAUR)	Receptor activity	NM_002659	2.5 *
Cell shape and actin cytoskeleton organization:			
CDC42 effector protein 3 (CDC42EP3)	Rho-GTPase binding	NM_006449	1.9
Coronin, actin binding protein, 2B (CORO2B)	Actin binding	NM_006091	1.7 *
CDC42 effector protein 2 (CDC42EP2)	Rho-GTPase binding	NM_006779	1.7
Podocalyxin-like (PODXL)	Intracellular cytoskeletal elements binding	NM_005397	1.6
Smoothelin (SMTN)	Actin binding	NM_134269	1.6 *
LIM domain kinase 2 (LIMK2)	Protein serine/threonine kinase activity	NM_005569	1.6
Disheveled associated activator of morphogenesis 1 (DAAM1)	Actin binding	NM_014992	1.5
Synaptopodin (SYNPO)	Actin binding	NM_007286	1.5
Paralemmin 2 (PALM2)	Kinase activity	NM_053016	1.5
Cell motility, cell migration and chemotaxis:			
Phosphatidic acid phosphatase type 2B (PPAP2B)	Phosphoprotein phosphatase activity	NM_003713	3.1 *
Plasminogen activator, urokinase receptor (PLAUR)	Receptor activity	NM_002659	2.5 *
Interleukin 8 (IL8)	Receptor binding	NM_000584	2.4 *
Plasminogen activator, urokinase (PLAU)	u-plasminogen activator activity	NM_002658	1.6 *

Synaptopodin (SYNPO)	Actin binding	NM_007286	1.5
Trophoblast glycoprotein (TPBG)	Transferase activity	NM_006670	1.5
Angiogenesis:			
Fms-related tyrosine kinase 1 (FLT1)	Receptor activity	NM_002019	2.7 *
Interleukin 8 (IL8)	Receptor binding	NM_000584	2.4 *
Jagged 1 (Alagille syndrome) (JAG1)	Growth factor activity	NM_000214	2.0 *
Vascular endothelial growth factor C (VEGFC)	Growth factor activity	NM_005429	1.8 *
Angiopoietin-like 4 (ANGPTL4)	Enzyme inhibitor activity	NM_016109	1.7 *
Chemokine (C-X-C motif) receptor 4 (CXCR4)	CXC chemokine receptor activity	NM_003467	1.6 *
Regulation of transcription:			
Early Growth Response 1(EGR1)	Transcription factor activity	NM_001964	3.6 *
Basic helix-loop-helix domain containing, class B, 2 (BHLHB2)	Transcription factor activity	NM_003670	2.6
High mobility group AT-hook 2 (HMGA2)	DNA binding	NM_003483	2.0
Coiled-coil domain containing 85B (CCDC85B)	DNA binding	NM_006848	1.8
Ets variant gene 5 (ets-related molecule) (ETV5)	Transcription factor activity	NM_004454	1.8
v-jun sarcoma virus 17 oncogene homolog (avian) (JUN)	Transcription factor activity	NM_002228	1.7
Kruppel-like factor 2 (lung) (KLF2)	Transcription factor activity	NM_016270	1.7

H2.0-like homeobox 1 (Drosophila) (HLX1)	Transcription factor activity	NM_021958	1.6
Hairy and enhancer of split 1, (Drosophila) (HES1)	DNA binding	NM_005524	1.5
Transcription factor 8 (TCF8)	Transcription co-repressor activity	NM_030751	1.5 *
Pleckstrin homology-like domain, family A, member 2 (PHLDA2)	DNA binding	NM_003311	1.5 *
CD3e molecule, epsilon associated protein (CD3EAP)	Protein binding	NM_012099	1.5
Intracellular Signaling Cascade:			
Dual specificity phosphatase 5 (DUSP5)	MAP Kinase phosphatase activity	NM_004419	3.0 *
Regulator of G protein signaling 2, 24kDa (RGS2)	Regulator of G protein signaling	NM_002923	2.2 *
Tribbles homolog 1 (Drosophila) (TRIB1)	Protein kinase inhibitor activity	NM_025195	2.2
Rap guanine nucleotide exchange factor (GEF) 3 (RAPGEF3)	Ras guanyl-nucleotide exchange factor activity	NM_012294	2.1
Protein tyrosine phosphatase, receptor Type, E (PTPRE)	transmembrane receptor protein tyrosine phosphatase activity	NM_006504	2.1
F-box and WD-40 domain protein 2 (FBXW2)	Ubiquitin protein ligase activity	NM_012164	2.0
Dual specificity phosphatase 4 (DUSP4)	Protein tyrosine/threonine phosphatase activity	NM_001394	2.0 *
Tribbles homolog 3 (Drosophila) (TRIB3)	Protein kinase binding	NM_021158	2.0
G-protein coupled receptor 4 (GPR4)	G-protein coupled receptor activity	NM_005282	1.8

A kinase (PRKA) anchor protein (gravin) 12 (AKAP12)	Protein kinase A binding	NM_005100	1.8 *
EPH receptor A4 (EPHA4)	Receptor activity	NM_004438	1.7 *
Ras interacting protein 1 (RASIP1)	Ras binding	NM_017805	1.7
Docking protein 5 (DOK5)	Adaptor protein	NM_018431	1.7 *
Cytokine-like 1 (CYTL1)	Receptor binding	NM_018659	1.7
Tensin 3 (TNS3)	Phosphoprotein phosphatase activity	NM_022748	1.7
LIM domain kinase 2 (LIMK2)	Protein serine/threonine kinase activity	NM_005569	1.6
START domain containing 8 (STARD8)	GTPase activator activity	NM_014725	1.6
Sprouty homolog 4 (Drosophila) (SPRY4)	Protein binding	NM_030964	1.6 *
Dual specificity phosphatase 6 (DUSP6)	MAP kinase phosphatase activity	NM_001946	1.6 *
SH2B adaptor protein 3 (LNK)	Lymphocyte adaptor protein	NM_005475	1.6
SH3-domain binding protein 5 (BTK-associated) (SH3BP5)	Protein kinase inhibitor activity	NM_004844	1.6
Malignant fibrous histiocytoma amplified sequence 1 (MFHAS1)	GTP binding	NM_004225	1.6
G protein-coupled receptor 116 (GPR116)	G-protein coupled receptor activity	NM_015234	1.5
Zinc finger, DHHC-type containing 13 (ZDHHC13)	Signal transducer activity	NM_019028	1.5

Cell metabolism and cell homeostasis:

Stanniocalcin 1 (STC1)	Hormone activity	NM_003155	4.6 *
Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (CHST2)	Transferase activity	NM_004267	3.5
Phosphatidic acid phosphatase type 2B (PPAP2B)	Phosphoprotein phosphatase activity	NM_003713	3.1 *
Lipase, endothelial (LIPG)	Lipoprotein lipase activity	NM_006033	2.5
Prostaglandin-endoperoxidase synthase 2 (PTGS2)	Prostaglandin endoperoxide synthase activity	NM_000963	1.8 *
Hexokinase 2 (HK2)	Hexokinase activity	NM_000189	1.8 *
GTP cyclohydroxylase 1 (dopa-responsive dystonia) (GCH1)	GTP cyclohydrolase I activity	NM_000161	1.8 *
Nucleoside phosphorylase (NP)	Purine-nucleoside phosphorylase activity	NM_000270	1.7
Membrane-associated ring finger (C3HC4) 3 (MARCH3)	Ubiquitin ligase activity	BC047569	1.7
ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3GAL6)	Sialyltransferase activity	NM_006100	1.7
Carbohydrate (chondroitin 4) sulfo-transferase 11 (CHST11)	Sulfotransferase activity	NM_018413	1.7
Coagulation factor II (thrombin) receptor-like 1 (F2RL1)	Receptor activity	NM_005242	1.7
Microtubule associated monoxygenase, calponin and LIM domain containing 2 (MICAL2)	oxidoreductase activity	NM_014632	1.6
Interferon stimulated exonuclease gene 20kDa (ISG20)	Exonuclease activity	NM_002201	1.5 *

Development:			
Four jointed box 1 (Drosophila) (FJX1)	Human ortholog of *Mus* and *Drosophila* FJX genes, function in humans unknown	NM_014344	1.8
Plexin A2 (PLXNA2)	Receptor activity	AB007932	1.7 *
Ion transport:			
Solute carrier family 4, sodium bicarbonate cotransporter, member 7 (SLC4A7)	Na+/HCO3- co-transporter activity	NM_003615	1.7
ATPase, Na+/K+ transporting, beta 1 polypeptide (ATP1B1)	Na+/K+ exchanging ATPase activity	NM_001001787	1.5
Miscellaneous genes:			
Leucine rich repeat containing 32 (LRRC32)	Transferase activity	NM_005512	1.7
Transmembrane protein 158 (TMEM158)	Role in Ras-induced senescence	NM_015444	1.7 *
B cell RAG associated protein (GALNAC4S-6ST)	Sulfotransferase activity	NM_015892	1.7
Leucine rich repeat containing 8 family, member 8 (LRRC8B)	Transferase activity	NM_015350	1.6
Similar to supported by human ESTs AA032221	Unknown function	LOC378137	1.6
Chromosome 3 open reading frame 40 (C3orf40)	Unknown function	NM_144635	1.5
Solute carrier family 43, member 3 (SLC43A3)	Transport of metabolites in rapidly growing or developing tissues	NM_014096	1.5
Neuron navigator 3 (NAV3)	ATPase activity	NM_014903	1.5

* Previously shown to be regulated in ECs by VEGF or during capillary morphogenesis in 3D collagen matrice

Table 2.3: Genes that were downregulated by Ang-1 exposure.

Gene (Symbol)	Molecular function(s)	Genbank ID	Fold reduction
Cell cycle and cell proliferation:			
Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (CDKN2C)	Cyclin-dependent protein kinase inhibitor activity	NM_001262	1.6
BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast) (BUB1)	Protein serine/threonine kinase activity	NM_004336	1.5
TTK protein kinase (TTK)	Protein kinase activity	NM_003318	1.5
Kinetochore associated 2 (KNTC2)	Spindle checkpoint signaling activity	NM_006101	1.5
Apoptosis:			
Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10)	Tumor necrosis factor receptor binding	NM_003810	2.0
Growth arrest and DNA-damage-inducible, beta (GADD45B)	Protein binding	NM_015675	1.7
Regulation of transcription:			
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2)	Transcriptional repressor activity	NM_002166	2.1
Zinc finger protein 323 (ZNF323)	Transcription factor activity	NM_145909	2.1
Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (ID3)	Transcriptional corepressor activity	NM_002167	1.7
Small nuclear RNA activating complex, polypeptide 1 (SNAPC1)	DNA binding	NM_003082	1.7
Bromodomain containing 8 (BRD8)	Ligand regulated transcription factor activity	NM_006696	1.7

Checkpoint suppressor 1 (CHES1)	Transcriptional repressor activity	NM_018589	1.6
Enhancer of zeste homolog 2 (Drosophila) (EZH2)	DNA binding	NM_004456	1.6
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1)	Transcriptional repressor activity	NM_002165	1.6
Runt-related transcription factor 1; translocated to, 1 (cyclin D-related) (RUNX1T1)	Transcription factor activity	NM_004349	1.5
Zinc finger protein 395 (ZNF395)	DNA binding	NM_018660	1.5
Signal transduction and intracellular Signaling Cascade:			
Epidermal growth factor receptor pathway substrate 8 (EPS8)	SH3/SH2 adaptor protein activity	NM_004447	1.9
Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (PIK3R3)	Phosphatidylinositol 3-kinase activity	NM_003629	1.9
Multiple C2 domains, transmembrane 1 (MCTP1)	Calcium ion binding	NM_024717	1.9
Serine/threonine kinase 38 like (STK38L)	Protein serine/threonine kinase activity	NM_015000	1.8
Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB)	Protein binding	NM_170662	1.7
A kinase (PRKA) anchor protein (yotiao) 9 (AKAP9)	Receptor-associated protein binding	NM_147171	1.6
Rho-guanine nucleotide exchange factor (RGNEF)	Rho guanyl-nucleotide exchange factor activity	NM_022448	1.6
Angiopoietin 2 (Ang-2 or ANGPT2)	Receptor binding	NM_001147	1.6
BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	Pseudoreceptor activity	NM_012342	1.5

(BAMBI)			
Development:			
Bone morphogenetic protein 4 (BMP4)	Signal transducer activity	NM_001202	2.3
Dachshund homolog 1 (Drosophila) (DACH1)	Protein binding	NM_080759	1.6
Metabolism:			
Nudix (nucleoside diphosphate linked moiety X)-type motif 4 (NUDT4)	Diphosphoinositol-polyphosphate diphosphatase activity (cyclic nucleotide metabolism)	NM_019094	1.6
Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)	Aldehyde dehydrogenase activity	NM_000689	1.6
Sulfotransferase family 1E, estrogen-Preferring, member 1 (SULT1E1)	Estrone sulfotransferase activity	NM_005420	1.6
N-myristoyltransferase 2 (NMT2)	Transferase activity	NM_004808	1.5
Sulfatase 1 (SULF1)	Aryl sulfatase activity	NM_015170	1.5
Multiple EGF-like-domains 9 (MEGF9)	Ca^{2+} ion binding	AB011542	1.5
Angiogenesis:			
Thrombospondin 1 (THBS1)	Signal transducer activity	NM_003246	1.5
Actin filament-based movement and cell shape:			
Myosin VI (MYO6)	Microfilament motor activity	NM_004999	1.6
Palmdelphin (PALMD)	Possible association with cytoskeletal structures	NM_017734	1.7
cell-cell signaling and cell-cell adhesion:			
Ephrin-A4 (EFNA4)	Transmembrane-ephrin receptor activity	NM_005227	1.6

FAT tumor suppressor homolog 4 (Drosophila) (FAT4)	Member of the cadherin superfamily	NM_024582	1.8
Immune response:			
Guanylate binding protein 1, interferon-inducible, 67kDa (GBP1)	GTP binding	NM_002053	1.7
Electron Transport:			
Cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1)	Monooxygenase activity	NM_019885	2.5
Peroxisome organization and biogenesis:			
Peroxisomal membrane protein 3 (PXMP3)	Protein binding	NM_000318	1.7
Phospholipid scrambling:			
Phospholipid scramblase 4 (PLSCR4)	Phospholipid scramblase activity	NM_020353	1.5
Establishment of tissue polarity:			
Frizzled homolog 2 (Drosophila) (FZD2)	Receptor activity	NM_001466	1.5
Nucleosome assembly:			
TSPY-like 4 (TSPYL4)	DNA binding	NM_021648	1.5
Response to heat:			
DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4)	Heat shock protein activity	NM_007034	1.6
Miscellaneous genes:			
Chromosome 8 open reading frame 4 (C8orf4)	Anchorage- independent growth	NM_020130	4.2

Serum deprivation response (SDPR)	Phosphatidylserine binding	NM_004657	2.5
Downregulated in ovarian cancer 1(DOC1)	Unknown function, possible role as anti-angiogenic molecule	NM_014890	2.0
Schlafen family member 12 (SLFN12)	Thymocyte maturation	NM_018042	1.6

RATIONALE FOR CHAPTER 3

Angiopoieitin-1 promotes *in vivo* and *in vitro* angiogenesis; however, the soluble molecules that mediate these effects of Ang-1 in ECs are not well characterized. In the previous chapter, we identified the gene expression profile that is induced in ECs upon treatment with Ang-1. The chemokine IL-8 was among the genes that were significantly upregulated in Ang-1-treated ECs. Since IL-8 is known to be synthesized and released by ECs and to promote migration and proliferation of these cells, we hypothesized that Ang-1 induces the production of IL-8 that acts in an autocrine manner to enhance EC proliferation and migration. In addition, we identified the mechanisms through which Ang-1 elicits induction of IL-8 expression in ECs.

CHAPTER 3

ANGIOPOIETIN-1 PROMOTES ENDOTHELIAL CELL PROLIFERATION AND MIGRATION THROUGH AP-1-DEPENDENT AUTOCRINE PRODUCTION OF INTERLEUKIN-8

Nelly A. Abdel-Malak[1], Coimbatore B. Srikant[2], Arnold S. Kristof[1], Sheldon A. Magder[1], John A. Di Battista[3] and Sabah N.A. Hussain[1]

[1] Critical Care and Respiratory Divisions, [2] Division of Endocrinology and [3] Division of Rheumatology, Department of Medicine, McGill University Health Centre, McGill University, Montreal, Quebec, Canada

Short Title: IL-8 and angiopoietin-1

Address of Correspondence: **Dr. S. Hussain,**
Room L3.05,
687 Pine Ave West,
Montréal, Québec, Canada H3A 1A1
sabah.hussain@muhc.mcgill.ca
Phone number : (514) 843-1664
Fax number : (514) 843-1686

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3.1. ABSTRACT

Angiopoietin-1 (Ang-1), ligand for the endothelial cell-specific Tie-2 receptors, promotes migration and proliferation of endothelial cells (ECs), however, whether these effects are promoted through the release of a secondary mediator remains unclear. In this study, we assessed whether Ang-1 promotes EC migration and proliferation through the release of interleukin-8 (IL-8). Ang-1 elicited in HUVECs a dose- and time-dependent increase in IL-8 production as a result of induction of mRNA and enhanced mRNA stability of IL-8 transcripts. IL-8 production is also elevated in HUVECs transduced with retroviruses expressing Ang-1. Neutralization of IL-8 in these cells with a specific antibody significantly attenuated proliferation and migration and induced caspase-3 activation. Exposure to Ang-1 triggered a significant increase in DNA binding of activator protein-1 (AP-1) to a relatively short fragment of IL-8 promoter. Upstream from the AP-1 complex, upregulation of IL-8 transcription by Ang-1 was mediated through the Erk1/2, SAPK/JNK and PI-3 kinase pathways, which triggered c-Jun phosphorylation on Ser^{63} and Ser^{73}. These results suggest that promotion of EC migration and proliferation by Ang-1 is mediated, in part, through the production of IL-8, which acts in an autocrine fashion to suppress apoptosis and facilitate cell proliferation and migration.

Key words: angiogenesis, Ang-1, IL-8, proliferation, migration

3.2. INTRODUCTION

Angiopoietin-1 (Ang-1) and its receptor, Tie-2, are rapidly emerging as important modulators of normal and pathological angiogenesis. In mice, deletion of Ang-1 gene produces lethality at E12.5, with major defects in the vascular endothelium[1]. In cultured ECs, Ang-1 inhibits apoptosis and inflammatory responses and promotes differentiation, sprouting and migration[2]. *In vivo*, Ang-1 enhances collateral vessel formation in ischemia-induced angiogenesis[3]. Exposure of ECs to Ang-1 triggers the autophosphorylation of Tie-2 receptors and the activation of downstream pathways, including the PI-3 kinase and three members of the mitogen activated protein kinases (MAPKs) (Erk1/2, p38 and SAPK/JNK)[2;4]. Whether these pathways mediate Tie-2 signaling through the release of soluble mediators remains unclear. Recently, two studies have reported that Ang-1 promotes smooth muscle cell (SMC) recruitment through the release of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), and hepatocyte growth factor (HGF) from ECs[5;6]. It remains unclear whether or not these secreted mediators, or as yet unknown factors, modulate Ang-1 effects on EC proliferation, apoptosis and migration.

We recently evaluated the transcriptome of HUVECs exposed to Ang-1 using microarrays [7]. Exposure to Ang-1 resulted in induction of 86 genes that are involved in EC proliferation, differentiation, migration and survival. In addition, Ang-1 elicited a significant decline in the expression of 49 genes, most of which are involved in cell-cycle arrest, apoptosis and suppression of transcription. Our results also revealed that the only proangiogenesis chemokine that was significantly induced by Ang-1 exposure in HUVECs was interleukin-8 (IL-8). IL-8 is a CXC chemokine with high binding affinity for CXCR1 and CXCR2 receptors, both of which are abundantly expressed on ECs. These results were surprising since Ang-1 exposure attenuates thrombin-induced IL-8 production in ECs [8]. IL-8 is mainly expressed by leukocytes, fibroblasts, ECs, and tumor cells and plays important roles in chemoattraction, inflammation, and tumour angiogenesis [9]. Others have reported that IL-8 directly induces EC migration, proliferation and tube formation [10]. These biological effects of IL-8, and our observation of an increase in IL-8 mRNA in HUVECs exposed to Ang-1, raise the possibility that some of the biological effects of Ang-1 on cultured ECs may be mediated indirectly

through the release of IL-8, which might act in an autocrine fashion on CXCR1 and CXCR2 receptors expressed on the surface of ECs. Accordingly, the main focus of the current study is to test the hypothesis that Ang-1-induced EC survival, migration and proliferation are mediated, in part, through increased IL-8 production. We also investigated in this study the signaling pathways and the transcription factors involved in the regulation of IL-8 production by the Ang-1/Tie 2 receptor pathway.

3.3. MATERIALS AND METHODS

Detailed methods can be found in Supplementary Material, section 3.8.

Cell culture

HUVECs were maintained in culture, as described [11]. Full-length murine Ang-1 cDNA was cloned into a retrovirus vector (MSCV-pac) and was transfected into Ampho Phoenix packaging cells. Viral supernatants from these cells were used to transduce HUVECs in multiple rounds of infection. Transduced cells were then selected in puromycin to produce MSCV-HUVECs and MSCV-Ang-1-HUVECs. For infection with adenoviruses, cells were infected overnight at a multiplicity of infection of 50 in serum-free medium.

Proliferation assays

For cell counting, cells were cultured in MCDB131 medium plus 2% fetal bovine serum (FBS). After 48 hours, cells were trypsinized and counted using a hemacytometer. For BrdU incorporation, cells were maintained in MCDB131 plus 2% FBS. After 24 hours, cells were pulsed with 10μM of BrdU and incubated for an additional 24 hours. Cells were then fixed, labeled, and absorbance was measured at 370nm using a BrdU Proliferation Assay (Roche, Indianapolis, IN).

RNA measurements

RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA), and IL-8 mRNA transcript was detected using Northern blotting and a non-radioactive cDNA probe corresponding to bases 47 to 344 of human IL-8 mRNA. Expression of Ang-1 and Ang-2

mRNA in retrovirally transduced HUVECs was measured with TaqMan® probes and a real-time polymerase chain reaction (PCR) apparatus (Applied Biosystems, Foster City, CA).

Immunoblotting and enzyme-linked immunosorbent assay

Total cell lysates, subcellular fractions and immunoprecipitated complexes were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and then probed with monoclonal and polyclonal antibodies. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents. IL-8 protein in culture medium was detected with commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

IL-8 promoter activity

HUVECs were transfected with firefly luciferase reporter plasmid driven by the -133/+44 bp segment of human IL-8 promoter and two additional plasmids in which the AP-1 binding element (-126 to -120 bp) and NFκB-like factor binding element (-80 to -71 bp) have been mutated (Document S1)[12]. Cells were treated with either solvent or 300 ng/ml of Ang-1, and firefly luciferase activity was measured using the Dual Luciferase Assay kit and normalized to the relative renilla luciferase activity.

Electrophoretic mobility shift assay:

Electrophoretic mobility shift assays (EMSAs) for NFκB and activator protein-1 (AP-1) were performed with a Gelshift NFκB/Rel and AP-1 Family (TPA-inducible) kits (Active Motif, Carlsbad, CA) employing double-stranded DNA probes for human NFκB and human IL-8/AP-1 (Document S1) and 5 to 10 μg of nuclear extracts, according to the manufacturer's instructions.

Wound healing:

Cell motility was assessed using the wound-healing assay as described previously [13]. Murine stem-cell virus (MSCV) or MSCV-Ang1 cells were grown for 24 hours in

basal medium. The media were collected and incubated at room temperature with anti-IL8 neutralizing antibody (7.5 μg/ml) or IgG control antibody (7.5 μg/ml). Fresh MSCV and MSCV-Ang-1 were seeded into 24-well tissue culture plates and were then carefully wounded using a 200-μl pipette tip. Cellular debris was removed by washing, and the media were then replaced with the conditioned media of MSCV and MSCV-Ang-1 cells. After 12 hours, the wound healing was visualized with inverted bright field microscopy and quantified.

Migration assay

Migration of HUVECs was performed in 24-well transwell polycarbonate inserts (8.0 μm pore size) of modified Boyden chambers, as described [14]. Conditioned media of MSCV or MSCV-Angl cells grown for 24 hours in basal medium were used as chemoattractants in the lower chamber. The apparatus was then incubated at 37°C in a CO_2 incubator for 5 hours, to allow for cell migration, then quantified, as described [14].

Statistics

Data were expressed as means plus or minus standard error (SE). Statistical significance was determined by one-way analysis of variance. Differences were considered statistically significant at P less than 0.05.

3.4. RESULTS

Regulation of IL-8 mRNA and protein by Ang-1

Ang-1 elicited significant, transient and concentration-dependent increases in EC IL-8 mRNA levels (Figures 3.1 A-C). In addition, Ang-1, but not Ang-2 or Ang-4, significantly induced IL-8 protein production in a time- and concentration-dependent fashion (Figure 3.1D). Similarly, vascular endothelial growth factor (VEGF) (80 ng/ml) induced a significant increased in IL-8 protein levels (Figure 3.1D). Ang-1-induced IL-8 protein was attenuated when HUVECs were pre-incubated with a soluble Tie-2 receptor protein (rhTie-2-FC) or the protein synthesis inhibitor cycloheximide (CHX) (Figure 3.1E).

Generation of retrovirally transduced human umbilical vein endothelial cells:

Previous studies have reported that Ang-1 elicits only a modest increase in EC proliferation. To augment the pro-proliferative properties of Ang-1 and to generate a cultured EC model that simulates the in vivo vasculature where Ang-1 is continuously being produced by vascular cells to stimulate EC Tie-2 receptors, we overexpressed Ang-1 in HUVECs, thereby allowing Ang-1 to strongly activate EC Tie-2 receptors in an autocrine fashion and stimulate EC proliferation. HUVECs were transduced with empty retroviruses (MSCV) or retroviruses expressing murine Ang-1 (MSCV-Ang-1). Real-time PCR confirmed the rise in murine Ang-1 mRNA expression in MSCV-Ang-1 cells (Figure 3.2A). Moreover, total Tie-2 protein levels decreased by 50%, and Tie-2 phosphorylation intensity increased by 90% in MSCV-Ang-1 compared with MSCV cells (Figure 3.2B). These results indicate strong activation of Tie-2 receptors in MSCV-Ang-1 cells. Ang-1 protein was detected mainly in the extracellular matrix (ECM) fraction of MSCV-Ang-1 cells, whereas relatively weaker Ang-1 protein levels were present in the media of these cells (Figure 3.2C). No Ang-1 protein was detected in the ECM and media of MSCV cells. Conditioned media of MSCV-Ang-1 cells, but not of MSCV cells, elicited a significant increase in Erk1/2 phosphorylation in control HUVECs (Figure 3.2D). In addition, media of MSCV-Ang-1 cells contained significantly greater IL-8 protein levels than that of MSCV cells (Figure 3.2E). Finally, cell number and BrdU incorporation over a 48-hour period were significantly greater, whereas cleaved caspase-3 intensities were significantly lower in MSCV-Ang-1 cells compared with MSCV cells (Figure 3.2 F,G). We concluded that secreted Ang-1 protein in MSCV-Ang-1 cells is biologically active, is incorporated mainly in the ECM, and promotes IL-8 production, EC proliferation, and attenuates apoptosis.

Roles of IL-8 in migration and proliferation:

To evaluate the role of IL-8 in EC migration, we performed wound healing and the modified Boyden migration assays. For wound healing, MSCV and MSCV-Ang-1 cells (donor cells) were serum starved for 24 hours, and conditioned media were then collected and incubated with 7.5 μg/ml of a monoclonal anti-IL-8 antibody or an isotype control antibody (control IgG). This concentration of anti-IL-8 antibody was chosen

based on successful blockade of IL-8 actions in ECs in previous studies [6]. In addition, we verified in additional experiments that anti-IL-8 antibody (7.5 μg/ml) completely blocked the rise in HUVEC proliferation and migration measured in response to exogenous recombinant human IL-8 (supplementary Figure S3.1). Fresh MSCV and MSCV-Ang-1 cells were wounded with a pipette tip and cultured for 12 hours in IgG-neutralized conditioned media (Figure 3.3A). Wound healing was significantly more rapid in MSCV-Ang-1 cells compared with MSCV cells, when both cell types were maintained in control IgG-neutralized condition media (Figure 3.3B). The healing rate of MSCV-Ang-1 cells incubated with IL-8-neutralized condition media was significantly lower than that measured with control IgG-neutralized media. By comparison, neutralization of IL-8 in the conditioned media did not alter the healing rate in control (MSCV) cells (Figure 3.3B). Figure 3B shows the changes in HUVEC (wild type) migration over a 5-hour period when conditioned media of MSCV or MSCV-Ang-1 cells were placed into the lower wells of modified Boyden chambers. Control IgG-neutralized conditioned media of MSCV-Ang-1 cells triggered significantly greater migration rates than did comparable conditioned media of MSCV cells ($P<0.05$). Neutralization of IL-8 exerted no effect on the chemoattractive capacity of MSCV cell conditioned media, but significantly attenuated that of MSCV-Ang-1 cells ($P<0.05$). These results suggest that IL-8 plays an important role in Ang-1-induced EC migration.

BrdU incorporation and cell number of MSCV-Ang-1 cells grown for 2 days in basal medium containing 2% FBS and control IgG antibody increased significantly, whereas the intensity of cleaved caspase-3 protein was significantly lower than in MSCV cells grown under similar conditions (Figure 3.3 C-E). For a given cell type, neutralization of IL-8 with anti-IL-8 antibody resulted in a significant increase in the intensity of cleaved caspase-3 compared with IgG control antibody (Figure 3.3 D,E). However, only in MSCV-Ang-1 cells did neutralization of IL-8 elicit in a decline in BrdU incorporation and cell number (Figure 3.3C).

Contribution of the PI-3 kinase and MAPK pathways

Ang-1 activates in ECs the PI-3 kinase and the Erk1/2, p38 and SAPK/JNK members of MAPKs [11;14]. Pre-incubation with selective inhibitors of the Erk1/2

(PD98059 and U0126 both at 30μM), the SAPK/JNK (15 μM SP600125) and the PI-3 kinase (50 nM wortmannin (WM) and 10 μM LY294002) pathways significantly attenuated Ang-1-induced IL-8 mRNA and protein levels (Figure 4). By comparison, pre-incubation with p38 pathway inhibitors (PD169316 and SB2030580, both at 10 μM) augmented Ang-1-induced IL-8 expression (Figure 3.5 A,B). This effect of p38 inhibitors was not altered by WM (Figure 3.5C), but was eliminated by PD98059 (Figure 3.5D). Thus, the rise in Ang-1-induced IL-8 mRNA expression by p38 inhibitors requires active Erk1/2, but not PI-3 kinase, pathway. These results also suggest that the p38 pathway inhibits Erk1/2 activation. This was confirmed by measuring the kinetics of Erk1/2 phosphorylation in response to Ang-1 in the absence and presence of SB203580. Figure 5E clearly indicates that Ang-1 exposure elicited a significant increase in Erk1/2 phosphorylation, which peaked after 15 minutes with a decline thereafter. However, when SB203580 (10 μM) was present in the culture medium, both the intensity and the duration of Erk1/2 phosphorylation elicited by Ang-1 were augmented. Moreover, overexpression of p38α protein in HUVECs elicited between 50 to 60% decline in Erk1/2 phosphorylation (Figure 3.5F). It should be noted that inhibition of Erk1/2 and p38 pathways had no effects on the degree of Ang-1-induced SAPK/JNK phosphorylation (supplementary figure S3.2). Similarly, inhibition of the SAPK/JNK pathway with SP600125 didn't influence Ang-1-induced Erk1/2 and p38 activation (results not shown), suggesting that the negative cross-talk between the p38 and Erk1/2 pathways doesn't involve the SAPK/JNK pathway.

IL-8 mRNA stability

To evaluate the contribution of changes in mRNA stability to Ang-1-induced IL-8 mRNA expression, HUVECs were exposed to Ang-1 for 1 hour. Cells were then washed and maintained for 2, 3 and 5 hours in fresh medium or fresh medium containing ActD alone or ActD plus Ang-1. When Ang-1 and ActD were not present in the culture medium, IL-8 mRNA levels declined rapidly (Figure 3.6A). Similarly, low levels of IL-8 mRNA were observed when ActD was present in the culture medium. However, when Ang-1 was present along with ActD, IL-8 mRNA levels were significantly greater than those measured with ActD alone (Figure 3.6 A,B), indicating that Ang-1 increases IL-8

mRNA stability. This effect of Ang-1 on IL-8 mRNA stability was eliminated when PD98059 (Erk1/2 inhibitor) was present along with Ang-1 (Figure 3.6 C,D). By comparison, IL-8 mRNA levels in cells maintained in medium containing ActD plus Ang-1 plus WM, LY294002 or SP600125 were not different from those measured in cells exposed to ActD plus Ang-1 (Figure 3.6E). These results indicate that Ang-1 promotes IL-8 mRNA stability through activation of the Erk1/2 and that the PI-3 kinase and SAPK/JNK pathways are not involved in this response.

Roles of AP-1 and NFκB in Ang-1-induced IL-8 production

Ang-1 elicited a significant rise in the activity of a relatively short fragment of human IL-8 promoter (-133/+44 bp; Figure 3.7A). This effect was eliminated by inhibition of the Erk1/2, SAPK/JNK and the PI-3 kinase pathways (Figure 7A). Furthermore, while mutation of the NFκB-like binding element (-80 to -71) had no effect on this response, Ang-1 failed to induce IL-8 promoter in which the AP-1 binding element (-126 to -120) was mutated (Figure 3.7A). Gel shift assays using oligonucleotides corresponding to AP-1 binding element of human IL-8 promoter revealed that Ang-1 significantly increased AP-1 DNA binding (225% ± 18%, Figure 3.7B). Supershift assays using antibodies specific for c-Jun and JunD impaired the formation or migration of AP-1/oligonucleotide complex both in vehicle- and Ang-1-treated cells, while antibodies for JunB, c-Fos, and FosB had no effect (Figure 3.7B). Moreover, Ang-1 treatment for 1 hour had no effect on mRNA expressions of Fos and Jun family members (supplementary figure S3.3). Similarly, protein levels of c-Fos, phospho-Fos (Thr^{325}), c-Jun and JunD remained unchanged by Ang-1 treatment (Figure 3.7C). Phosphorylation of c-Jun on Ser^{63} and Ser^{73} are critical for transactivation of this protein and the rise in AP-1 DNA binding. Ang-1 induced a significant increase in c-Jun phosphorylation on Ser^{63} and Ser^{73} (Figure 3.7C). This effect was attenuated by Erk1/2 and SAPK/JNK inhibitors but not by PI-3 kinase inhibitors (Figure 3.7D). Thus, Ang-1 treatment triggered an increase in c-Jun phosphorylation and enhanced AP-1 DNA binding to IL-8 promoter. These responses were associated with increased IL-8 promoter activity and IL-8 protein production (Figure 3.8).

We excluded the involvement of NFκB in Ang-1-induced IL-8 production in HUVECs because Ang-1 had no effect on total IκB, phospho (Ser^{536}) p65 levels and nuclear mobilization of the p65 and the p50 subunits of NFκB and had no influence on NFκB binding to DNA probe (supplementary figure S3.4). Finally, transduction of HUVECs with adenoviruses expressing dominant negative forms of IKK-α, or IKK-β failed to attenuate Ang-1-induced IL-8 expression (supplementary figure S3.4).

3.5. DISCUSSION

The main findings of this study are as follows: (1) Ang-1 induces significant increases in IL-8 production in HUVECs through induction of transcription and enhanced IL-8 mRNA stability; (2) the Erk1/2, SAPK/JNK, and PI-3 kinase pathways enhance IL-8 transcription in response to Ang-1, primarily through regulation of c-Jun phosphorylation and AP-1 binding to the IL-8 promoter, whereas increased IL-8 mRNA stability is mediated through the Erk1/2 pathway; (3) the p38 MAPK pathway exerts a negative influence on IL-8 production, a result of selective inhibition of Erk1/2 pathway activation; and (4) autocrine IL-8 effects are required for Ang-1-induced EC wound healing, migration, and proliferation.

We report here that Ang-1 induces the production of IL-8 in ECs, which acts in an autocrine fashion to enhance migration and proliferation of these cells. IL-8 is secreted by a variety of cells, including ECs, in response to cytokines, viruses and oxidants. Previous studies have revealed that Ang-2 has no effect on IL-8 production in myelogenous leukemia blasts [15]. By comparison, VEGF provokes a rapid induction of IL-8 mRNA in ECs, followed by a sustained increase in IL-8 protein levels [16]. We have confirmed that VEGF induces IL-8 production in HUVECs (Figure 3.1). In this respect, induction of IL-8 expression in HUVECs by Ang-1 is qualitatively similar to that triggered by VEGF.

In contrast to our finding of an increase in IL-8 production in ECs by Ang-1, Pizurki *et al.* [8] have recently reported that Ang-1 attenuates thrombin-induced IL-8 production in EA.hy926 cells (hybrid ECs)[8]. We speculate that the discrepancies between our study and that of Pizurki *et al.* are related to the cellular context and the nature of transcription factors involved in the induction of IL-8. For instance, upregulation of IL-8 production by Ang-1 was mediated mainly through AP-1 binding, with the Erk1/2,

SAPK/JNK, and PI-3 kinase pathways being upstream regulators. In comparison, thrombin induces substantial levels of IL-8 through activation of the p38 MAPK pathway and NFκB transcription factor [17;18]. Hughes *et al.* [19] have demonstrated that Ang-1 inhibits NFκB activation through selective interaction with ABIN-2 (a novel inhibitor of NFκB). This finding could explain the inhibitory effect of Ang-1 on thrombin-induced IL-8 production reported by Pizurki *et al.* In addition, whereas the p38 MAKP pathway promotes IL-8 production when thrombin is present [18], it inhibits IL-8 production in the presence of Ang-1 alone (Figure 3.5). These results suggest that the Ang-1/Tie-2 pathway might exert two opposing effects on IL-8 production, depending on the cellular context and the molecular mechanisms involved in the induction of the IL-8 promoter.

IL-8 gene expression is regulated through transcriptional activation and enhanced mRNA stability [20]. Both of these mechanisms were invoked by Ang-1. Increased IL-8 mRNA stability by Ang-1 is consistent with previous reports documenting that cytokines (IL-1β and TNF-α), and growth factors (IGF-II and VEGF) enhance IL-8 mRNA expression, in part through increased mRNA stability [21]. With respect to IL-8 transcription, many reports have identified NFκB and AP-1 transcription factors as the main regulators of IL-8 promoter activity in response to a variety of stimuli. In the current study, we have confirmed that Ang-1 triggers a significant rise in the activity of a relatively short fragment of human IL-8 promoter and that mutation of the AP-1 binding element in this portion (-126 to -120) abrogated this response (Figure 3.7). The involvement of AP-1 in Ang-1-induced IL-8 production was further confirmed by using gel shift assays (Figure 3.7). We should point out that because the influence of Ang-1 treatment on the activity of the full human IL-8 promoter wasn't assessed in this study, one could not exclude the involvement of other transcription factors other than AP-1 and other modulators of IL-8 transcription such as reactive oxygen species in the induction of IL-8 production by the Ang-1/Tie-2 receptor pathway in ECs.

AP-1 transcription factors are composed of Jun (c-Jun, JunB, and JunD) family homodimers, Jun/Fos (c-Fos, FosB, Fra1, and Fra2) or Jun/ATF2 heterodimers. Abundance and phosphorylation of transactivation domains of these proteins regulate AP-1 transcriptional activity. We found that c-Jun phosphorylation on Ser^{63} and Ser^{73} was significantly elevated by Ang-1 exposure. c-Jun consists of a C-terminal DNA-

binding/leucine zipper domain and an N-terminal trans-activation domain. De-phosphorylation of serine and threonine residues in the C-terminal of c-Jun is necessary for DNA binding, while phosphorylation on Ser^{63} and Ser^{73} in the N-terminal promotes AP-1 transactivation [22]. Our observations that c-Jun phosphorylation at Ser^{63} and Ser^{73} was enhanced in response to Ang-1, and that the AP-1 complex bound to IL-8 DNA consisted mainly of c-Jun, suggest that c-Jun plays an important role in Ang-1-induced IL-8 production. One pathway responsible for increased c-Jun phosphorylation downstream from Tie-2 receptor is the SAPK/JNK pathway, which directly phosphorylates c-Jun on Ser^{63} and Ser^{73} [23]. We also found that Ang-1 triggers c-Jun phosphorylation through the Erk1/2 pathway (Figure 3.7D). It should be noted that this response is not dependent on a cross-talk between Erk1/2 and with SAPK/JNK pathways, as Erk1/2 pathway inhibition had no effect on Ang-1-induced SAPK/JNK phosphorylation (supplementary figure S3.2). One likely mechanism through which the Erk1/2 proteins regulate c-Jun phosphorylation is a direct action in which c-Jun might serve as a substrate for Erk proteins, as recently documented in embryonic fibroblasts [24]. Finally, we report here that Ang-1 promotes IL-8 production, in part through the PI-3 kinase pathway. The exact molecular mechanisms involved in this response remain unclear. The observation that inhibition of PI-3 kinase pathway by wortmannin had no effects on c-Jun phosphorylation suggests that this pathway regulates IL-8 transcription through transcription factors other than AP-1 (Figure 3.7). The nature of these factors remains to be determined. Another possibility is that the PI-3 kinase pathway promotes IL-8 production by activating the Erk1/2 pathway as described previously [11]. It is also possible that the PI-3 kinase pathway may promote AP-1 activity and IL-8 induction through inactivation of negative regulators of c-Jun DNA binding activity. One such regulator is glycogen synthase kinase 3 (GSK3), which phosphorylates c-Jun at C-terminal sites and inhibits the binding activity of this protein [25]. By phosphorylating and inactivating GSK3 through protein kinase B (AKT), the PI-3 kinase pathway might trigger an increase in c-Jun DNA binding activity, leading eventually to increased IL-8 transcription.

Unlike the Erk1/2, SAPK/JNK and PI-3 kinase pathways, the p38 MAPK pathway inhibits Ang-1-induced IL-8 production, an effect that is mediated through

selective attenuation of Erk1/2 pathway activation. This negative cross-talk between the p38 and Erk1/2 pathways is not unique to ECs, nor to the Ang-1/Tie-2 receptor pathway [26]. In the case of the Ang-1/Tie-2 receptor pathway, the inhibitory role of p38 MAPKs may function as a biological switch through which other stimuli are able to modulate the degree to which the Ang-1/Tie-2 receptor pathway induces IL-8 and, by extension, EC proliferation and migration.

IL-8 is a chemoattractant for neutrophils and T lymphocytes and has been associated with inflammatory cell infiltration in many disease states. In addition, many reports have described important roles for IL-8 in embryonic and adult vascular formation and in tumor angiogenesis [27]. The importance of IL-8 in tumor growth is mediated through both increased recruitment of inflammatory cells to the site of the tumors and through direct effects of IL-8 on ECs [28]. In cultured ECs, IL-8 elicits angiogenic activities manifested by increased proliferation, migration and *in vitro* capillary tube formation [10]. It also inhibits apoptosis and induces MMP-2 production through activation of both CXCR1 and CXCR2 receptors in HUVECs [10]. In the present study, we assessed the role of IL-8 in Ang-1-induced migration by using conditioned media of MSCV and MSCV-Ang-1 cells neutralized with anti-IL-8 or control IgG antibodies. Conditioned media of MSCV-Ang-1 neutralized with control IgG antibody elicited significantly faster healing rates in wound assays and greater EC migration in Boyden chamber assays, as compared with comparable media of MSCV cells. This suggests that Ang-1 elicits the release of soluble factors from MSCV-Ang-1 cells that promote EC migration. That IL-8 is one of these factors is indicated by the observation that neutralization of IL-8 results in disappearance of the pro-migratory effects of MSCV-Ang-1 conditioned media (Figure 3.3B). The results shown in Figure 3C also indicate that, in addition to promoting migration, IL-8 contributes, in part, to the enhancement of proliferation in MSCV-Ang-1. The roles played by IL-8 in Ang-1-induced EC migration and proliferation, therefore, are consistent with previous reports documenting that IL-8 acts in an autocrine fashion to promote migration and proliferation in cultured ECs [10]. We should emphasize that even after IL-8 neutralization, cell number and BrdU incorporation in MSCV-Ang-1 cells remains higher than it does in MSCV cells, suggesting that factors other than IL-8 also contribute to the pro-proliferative effects of Ang-1.

We also assessed the contribution of IL-8 to the anti-apoptotic properties of Ang-1 by comparing cleaved caspase-3 intensities in MSCV and MSCV-Ang-1 cells. We observed a significant increase in cleaved caspase-3 intensity in both cell types when IL-8 was neutralized with a selective antibody (Figure 3.3D,E). These results are consistent with the notion that IL-8 functions as an autocrine regulator of EC survival through activation of CXCR1 and CXCR2 receptors located on these cells. Promotion of EC survival by IL-8 is believed to be through upregulation of the anti-apoptotic members of the Bcl-2 family of proteins, including Bcl-2 and Bcl-XL [29]. That cleaved caspase-3 intensity after IL-8 neutralization in MSCV-Ang-1 cells remained lower than that in MSCV cells suggests that additional mechanisms, other than IL-8, contribute to the anti-apoptotic effects of Ang-1 in MSCV-Ang-1 cells. As it has been well established that the Ang-1/Tie-2 receptor pathway regulates EC survival through several mechanisms, including induction of Survivin-1 protein, activation of the Erk1/2 and PI-3 kinase pathways, and inhibition of the release of mitochondrial activators of caspases (Smac) [2], any or all of these mechanisms might have contributed to the relatively lower levels of cleaved caspase-3 intensities in MSCV-Ang-1 cells.

We should emphasize that the importance of IL-8 in the *in vivo* functions of the Ang-1/Tie-2 receptor pathway, particularly the critical role of this pathway in embryonic vascular development, remains to be investigated. Documenting this importance particularly, in developing embryos, may prove to be difficult because of the well-described redundancy and overlap in the biological effects of chemokines and their receptors. For instance, genetic deletion of CXCR2, the main receptor for the murine chemokine equivalent of IL-8, is associated with significant upregulation of CXCR1 [30], suggesting that the absence of major vascular defects in $CXCR2^{-/-}$ mice might have been prevented as a consequence of compensatory increase in CXCR1 expression [31].

In summary, we report here that Ang-1 elicits significant IL-8 production in HUVECs as a result of increased IL-8 transcription and improved IL-8 mRNA stability. These effects are mediated through activation of the Erk1/2, SAPK/JNK and PI-3 kinase pathways, with AP-1 being the downstream transcription factor. Finally, enhanced IL-8 production plays important roles in mediating Ang-1-induced EC migration and proliferation.

3.6. ACKNOWLEDGMENT

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Authorship: N. Abdel-Malak designed and performed research, analyzed data and wrote the paper; C. B. Srikant contributed new analytical tools and designed research; A. S. Kristof provided new analytical tools and contributed new reagents; S. Magder performed research; J. A. Di Battista provided new reagents and analytical tools; S. N.A. Hussain designed research and wrote the paper.

Conflict of interest disclosure: All authors declare no conflicts of interest or financial interests.

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Figure 3.1: Ang-1 induces expression of IL-8 mRNA and protein.

A: A representative IL-8 Northern blot. HUVECs were serum starved for 12 hours and were then stimulated with Ang-1 (300 g/ml) and collected 1, 2, 3 and 6 hours later. C indicates control samples, 18S refers to rRNA.

B: Means (± SEM; n=3) of IL-8 mRNA intensity triggered by 300 ng/ml of Ang-1.

C: Means (± SEM; n=3) of IL-8 mRNA intensity measured after 1 hour of Ang-1 addition.

D: Means (± SEM; n=6) of IL-8 protein (detected with ELISA) in culture medium in response to Ang-1, Ang-2 and Ang-4 (300 ng/ml) and VEGF (80 ng/ml).

E: Means (± SEM; n=6) of IL-8 protein measured after 12 hours of Ang-1 (100%) or Ang-1 plus rhTie2-FC protein (100x in excess of Ang-1) or Ang-1 plus cycloheximide (50 μg/ml, CHX). For panels C and D, $*P<0.05$ compared with control values. For panel E, $*P<0.05$ compared with Ang-1 alone.

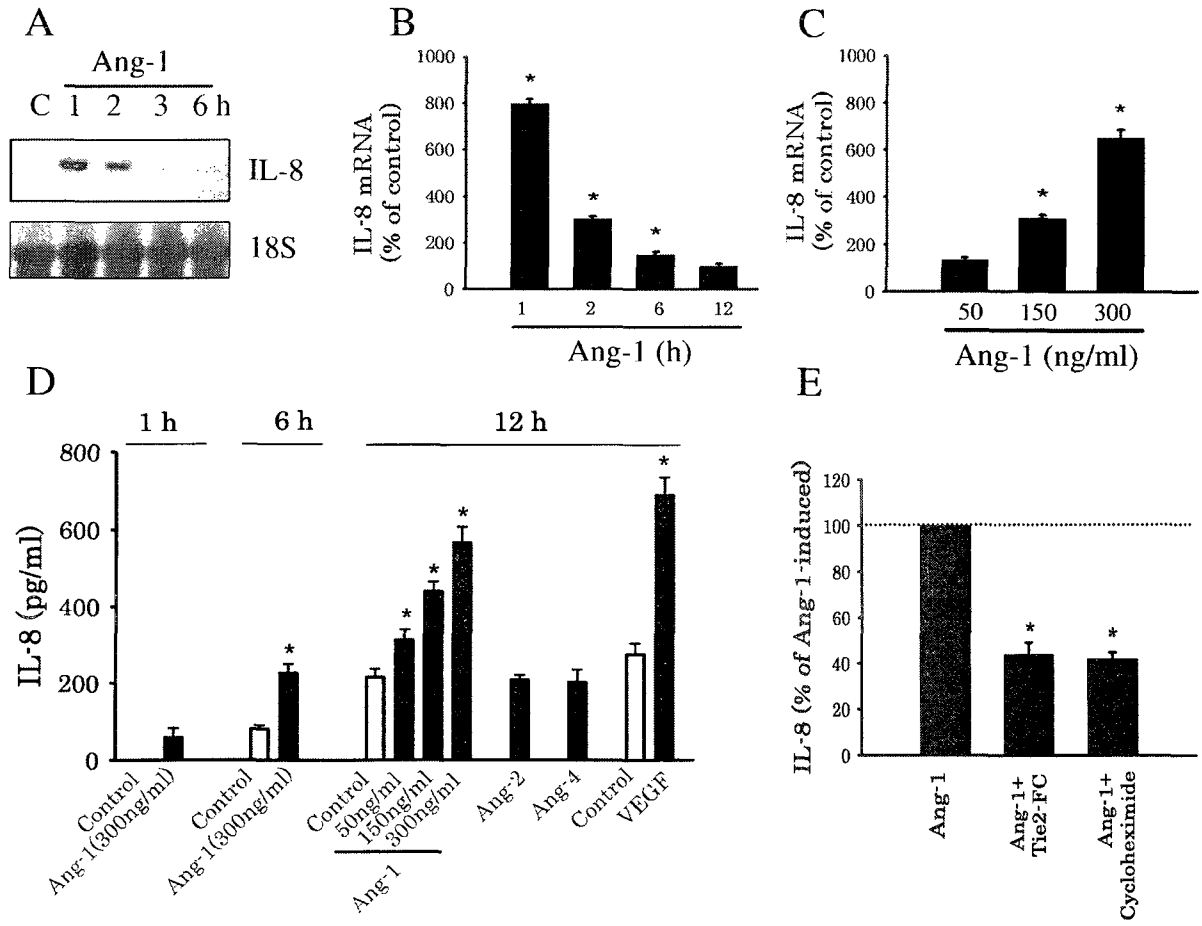

A
Ang-1
C 1 2 3 6 h
IL-8
18S
B
IL-8 mRNA (% of control)
1000
800
600
400
200
0
1 2 6 12
Ang-1 (h)
C
IL-8 mRNA (% of control)
1000
800
600
400
200
0
50 150 300
Ang-1 (ng/ml)
D
1 h
6 h
12 h
IL-8 (pg/ml)
800
600
400
200
0
Control
Ang-1(300ng/ml)
Control
Ang-1(300ng/ml)
Control
50ng/ml
150ng/ml
300ng/ml
Ang-1
Ang-2
Ang-4
Control
VEGF
E
IL-8 (% of Ang-1-induced)
120
100
80
60
40
20
0
Ang-1
Ang-1+ Tie2-FC
Ang-1+ Cycloheximide

FIGURE 3.1

Figure 3.2: Generation of HUVECs overexpressing murine Ang-1 using a retroviral vector.

A: Expression of murine Ang-1 and human Ang-2 mRNA levels in HUVECs transduced with control retroviruses (MSCV) and retroviruses expressing murine Ang-1 (MSCV-Ang-1). $^{**}P<0.01$ compared with MSCV cells.

B: Immunoblotting of MSCV and MSCV-Ang-1 cell lysates for Tie-2, tyrosine phosphorylated Tie-2 (p-Tie-2), V5 tag and Tie-1 proteins.

C: Detection of Ang-1 protein in the medium and extracellular matrix (ECM) of MSCV and MSCV-Ang-1 cell using immunoblotting.

D: Erk1/2 phosphorylation measured after 15 minutes' exposure to conditioned medium of MSCV and MSCV-Ang-1 cells. HUVECs were cultured in complete medium overnight and were then serum starved for 6 hours. The medium was then removed and replaced by conditioned medium collected from serum starved MSCV and MSCV-Ang-1 cells.

E: Detection of IL-8 protein in the medium of MSCV and MSCV-Ang-1 cells using ELISA (n=6). $^{*}P<0.05$ compared with MSCV cells.

F,G: Means (± SEM; n=6) of cell number, BrdU incorporation and cleaved caspase-3 intensity after 2 days' culturing in culture medium containing 2%FBS. $^{*}P<0.05$ compared with MSCV cells.

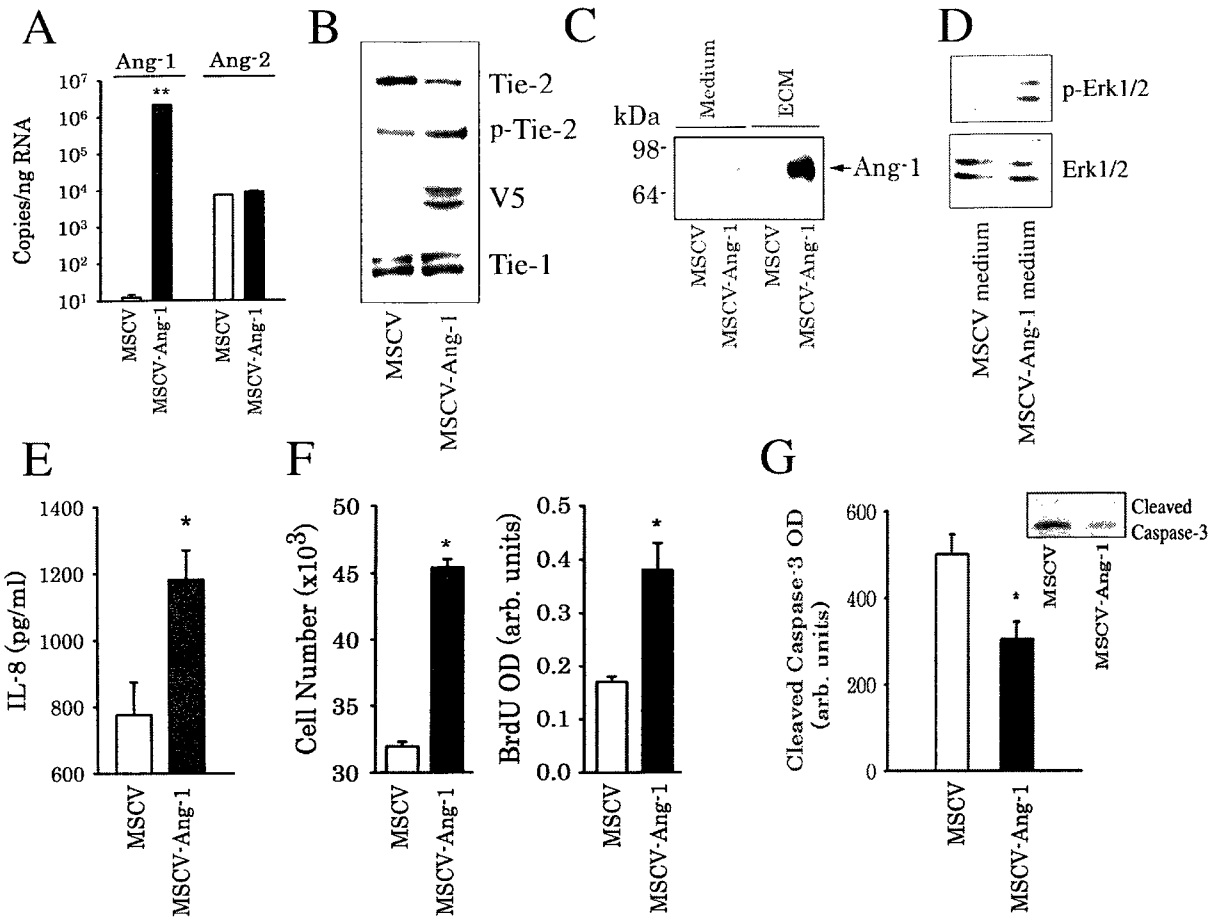

A
Ang-1
Ang-2
Copies/ng RNA
MSCV
MSCV-Ang-1
B
Tie-2
p-Tie-2
V5
Tie-1
C
kDa
Medium
ECM
98-
64-
Ang-1
D
p-Erk1/2
Erk1/2
MSCV medium
MSCV-Ang-1 medium
E
IL-8 (pg/ml)
F
Cell Number (x10^3)
BrdU OD (arb. units)
G
Cleaved Caspase-3 OD (arb. units)
Cleaved Caspase-3

FIGURE 3.2

Figure 3.3: Ang-1-induced IL-8 plays a significant role in EC migration and proliferation.

A: Representative examples of MSCV and MSCV-Ang-1 cell wounding experiments. MSCV (left panels) and MSCV-Ang-1 cells (right panels) were wounded (time 0) and maintained for 12 hours in conditioned media derived from MSCV and MSCV-Ang-1 cells, respectively, and were neutralized with IgG control antibody. Arrows point to the edges of the wounds. Note that wound healing (measured after 12 hours) was faster in MSCV-Ang-1 cells compared with MSCV cells.

B, top panel: Mean (± SEM; n=6) of wound healing in MSCV and MSCV-Ang-1 cells maintained for 12 hours in conditioned media of donor MSCV and MSCV-Ang-1 cells, respectively. MSCV or MSCV-Ang-1 cells were grown for 24 hours in basal medium. The media was collected and incubated with anti-IL-8 neutralizing antibody or IgG control antibody. Fresh MSCV and MSCV-Ang-1 cells were seeded into 24-well tissue culture plates and cultured in complete medium containing 20% FBS to nearly confluent cell monolayers. The cells were then carefully wounded using a pipette tip. After making the wounds, culture media were replaced with conditioned media of MSCV and MSCV-Ang-1 cells neutralized with anti-IL-8 or control IgG antibody. Wounds were then photographed (time = 0 and 12 hours later). Migration was evaluated by measuring the reduction in the diameter of the wound after migration of the cells into the cell-free zone. $*P<0.05$ compared with MSCV cells maintained in IgG-neutralized conditioned media. $\#P<0.05$ compared with MSCV-Ang-1 maintained in IgG-neutralized conditioned medium.

B, bottom panel: Means (± SEM; n=6) of the number of HUVECs migrating toward conditioned media derived from MSCV or MSCV-Ang-1 cells. EC migration was performed in 24-well transwell fibronectin-coated polycarbonate inserts. HUVECs were suspended in basal medium and seeded in the upper compartment. MSCV or MSCV-Ang-1 cells were grown in basal media for 24 hours, and conditioned media were then collected and incubated with anti-IL-8 and control IgG antibodies. Conditioned media

were then placed into the lower compartment of the migration apparatus. Migration was quantified 5 hours later by counting cells in 10 fields per well. Symbols are the same as in B top panel.

C: BrdU incorporation and cell number of MSCV and MSCV-Ang-1 cells maintained for 2 days in basal medium containing 2%FBS and IgG or anti-IL-8 antibodies. $*P<0.05$ compared with MSCV cells maintained in the presence of IgG antibody. $\#P<0.05$ compared with MSCV-Ang-1 cells maintained in the presence of IgG antibody.

D,E: A representative immunoblot of cleaved caspase-3 and means (± SEM; n=4) of cleaved caspase-3 intensity measured in MSCV and MSCV-Ang-1 cells after 2 days of culture in basal medium containing 2%FBS and IgG or anti-IL-8 antibodies. Symbols are the same as in panel B.

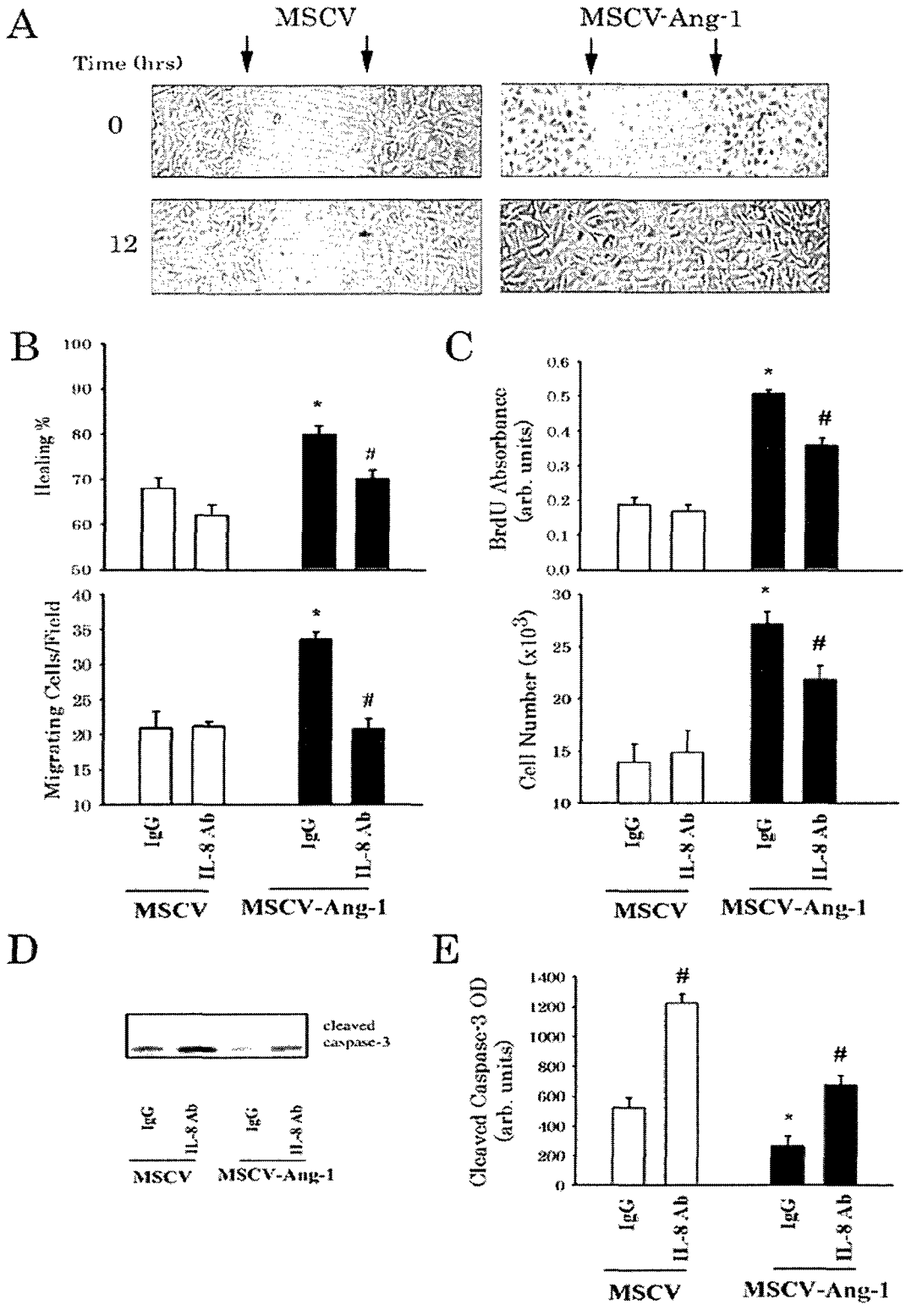

A
MSCV
MSCV-Ang-1
Time (hrs)
0
12
B
Healing %
Migrating Cells/Field
C
BrdU Absorbance (arb. units)
Cell Number (x10^3)
IgG
IL-8 Ab
MSCV
MSCV-Ang-1
D
cleaved caspase-3
E
Cleaved Caspase-3 OD (arb. units)

FIGURE 3.3

Figure 3.4: Roles of the PI-3 kinase and the SAPK/JNK and Erk1/2 MAPK pathways in Ang-1-induced IL-8 mRNA and protein expression.

A,B: Effects of wortmannin (WM, 50 nM), LY294002 (10 μM), SP600125 (15 μM) and PD98059 (30 μM) on IL-8 mRNA levels (representative blots in panel A and means [± SEM] in panel B) measured after 1 hour of Ang-1 exposure.

C: Mean (± SEM; n=3) of IL-8 protein measured after 12 hours of Ang-1 (expressed as 100%) or Ang-1 plus U0126 (30 μM), PD98059 (30 μM), LY294002 (10 μM) or SP600125 (15 μM). *P<0.05 compared with Ang-1 alone.

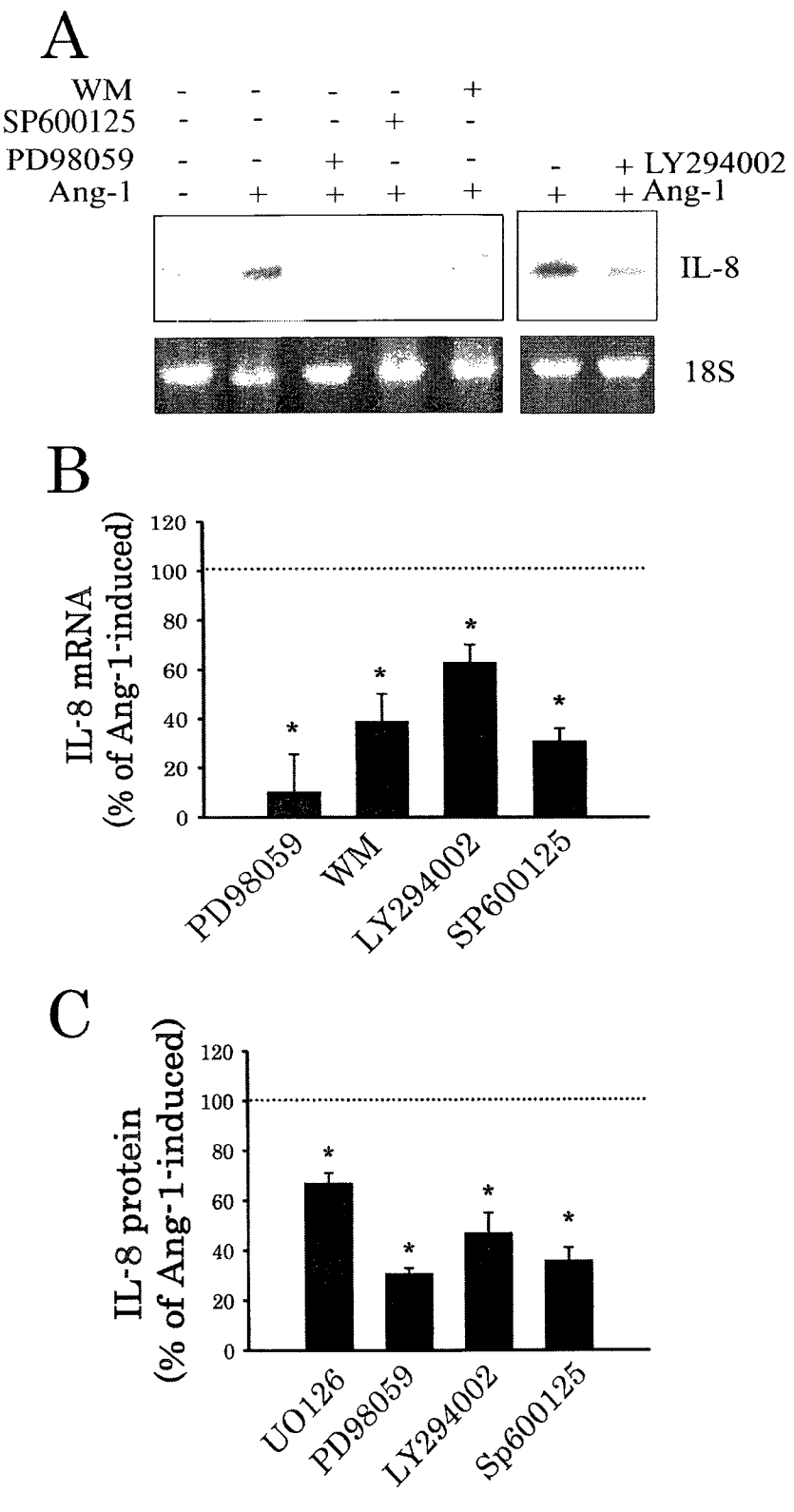

A
WM - - - - +
SP600125 - - - + -
PD98059 - - + - -
Ang-1 - + + + +
- + LY294002
+ + Ang-1
IL-8
18S
B
IL-8 mRNA (% of Ang-1-induced)
120
100
80
60
40
20
0
PD98059
WM
LY294002
SP600125
C
IL-8 protein (% of Ang-1-induced)
120
100
80
60
40
20
0
UO126
PD98059
LY294002
Sp600125

FIGURE 3.4

Figure 3.5: Role of the p38 MAPK in Ang-1-induced IL-8 expression.

A,B: Representative Northern blot and means (± SEM; n=3) of IL-8 mRNA levels measured after 1 hour of Ang-1 (expressed as 100%) and Ang-1 plus PD169316 or SB203580 (both at 10 μM). *$P<0.05$ compared with Ang-1 alone.

C: Representative Northern blot of IL-8 mRNA expression measured after 1 hour of Ang-1 and Ang-1 plus PD169316 (10 μM), WM (50 nM) or a combination of the two.

D: Representative Northern blot of IL-8 mRNA expression measured after 1 hour of Ang-1 and Ang-1 plus PD169316 (10 μM), PD98059 (30 μM) or a combination of the two.

E: Influence of p38 inhibition on Ang-1-induced Erk1/2 phosphorylation. Serum starved HUVECs were incubated for 1 hour with SB203580 (10 μM) and were then stimulated with Ang-1 (300 ng/ml) for 5, 15, 30 and 60 minutes. Cells were then collected and the levels of phosphorylation and total Erk1/2 proteins were detected with immunoblotting. Note the increase in both the intensity and duration of Erk1/2 phosphorylation when SB203580 was present in the medium.

F: Total and phosphorylated Erk1/2, Flag and p38α proteins detected in HUVECs mock-transfected or transfected with Flag-p38α palsmid.

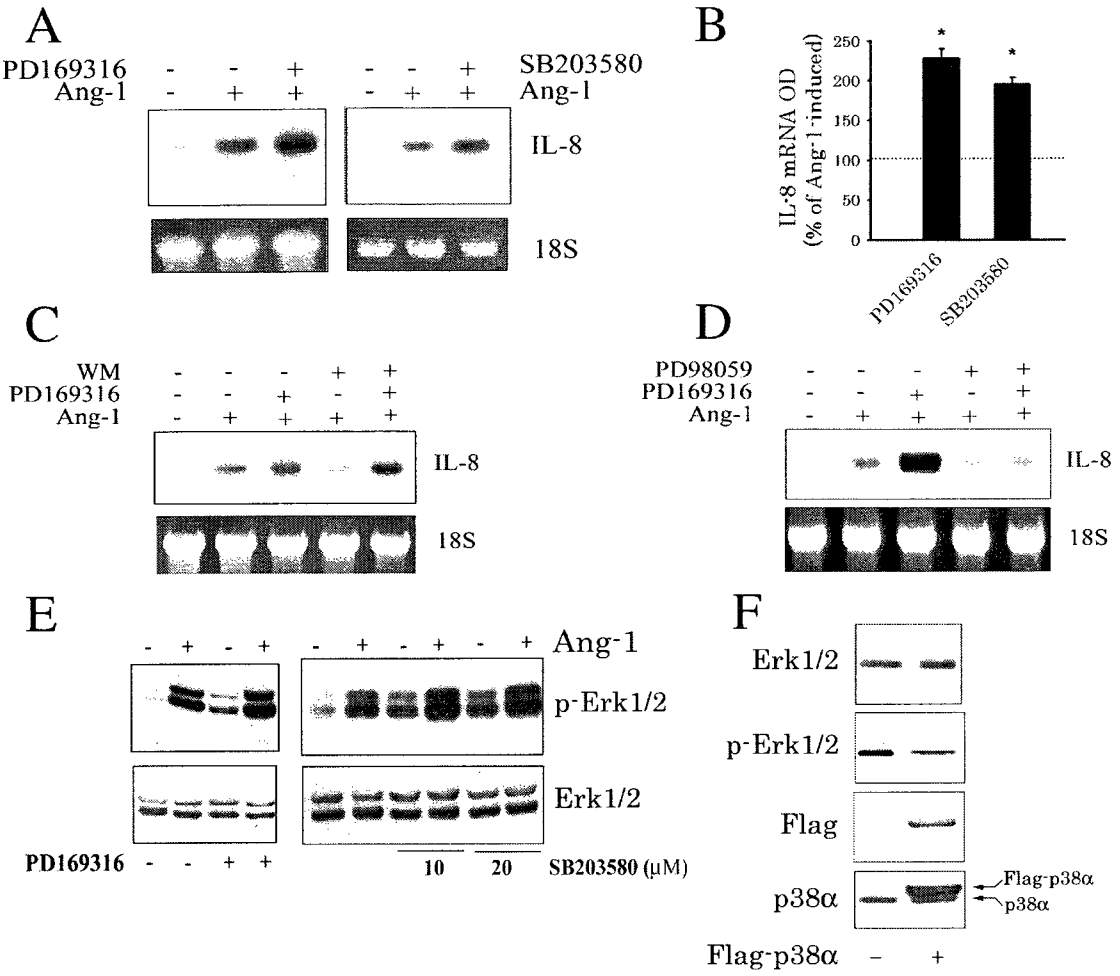

A
PD169316
Ang-1
SB203580
Ang-1
IL-8
18S
B
IL-8 mRNA OD
(% of Ang-1-induced)
250
200
150
100
50
0
PD169316
SB203580
C
WM
PD169316
Ang-1
IL-8
18S
D
PD98059
PD169316
Ang-1
IL-8
18S
E
Ang-1
p-Erk1/2
Erk1/2
PD169316
10
20
SB203580 (μM)
F
Erk1/2
p-Erk1/2
Flag
p38α
Flag-p38α
p38α
Flag-p38α

FIGURE 3.5

Figure 3.6: Ang-1 enhances IL-8 mRNA stability through Erk1/2 activation.

A: Cells were first exposed for 1 hour to Ang-1 and were then maintained in fresh medium, medium containing 5 μg/ml ActD (- Ang-1 + ActD), or ActD and Ang-1 (300 ng/ml; + Ang-1 + ActD). Cells were then collected after different time periods. Total RNA was then extracted, and IL-8 and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA (controls) levels were detected with Northern blotting.

B: Means (± SEM; n=3) of IL-8 mRNA intensities measured in cells undergoing protocols shown in panel A. mRNA intensities expressed as percentage of those measured after 1 hour of Ang-1 treatment. $*P<0.05$ compared with - Ang-1 + ActD.

C,D: Representative Northern blot and means (n=3) of IL-8 mRNA. Cells were treated with Ang-1 for 1 hour and were then maintained in medium containing ActD plus Ang-1 (+ Ang-1 + ActD) and Ang-1 plus ActD + PD98059 (30 μM). Cells were then collected after different time periods, and total RNA was extracted and underwent Northern blotting for IL-8 and 18S levels.

E: Mean values (± SEM; n=3) of IL-8 mRNA measured in cells which were treated first with Ang-1 for 1 hour (100% values) and were then maintained for an additional 1 hour in media containing Ang-1 plus ActD, Ang-1 plus ActD plus WM (50 nM), Ang-1 plus ActD plus LY294002 (10 μM) and Ang-1 plus ActD plus SP600125 (15 μM).

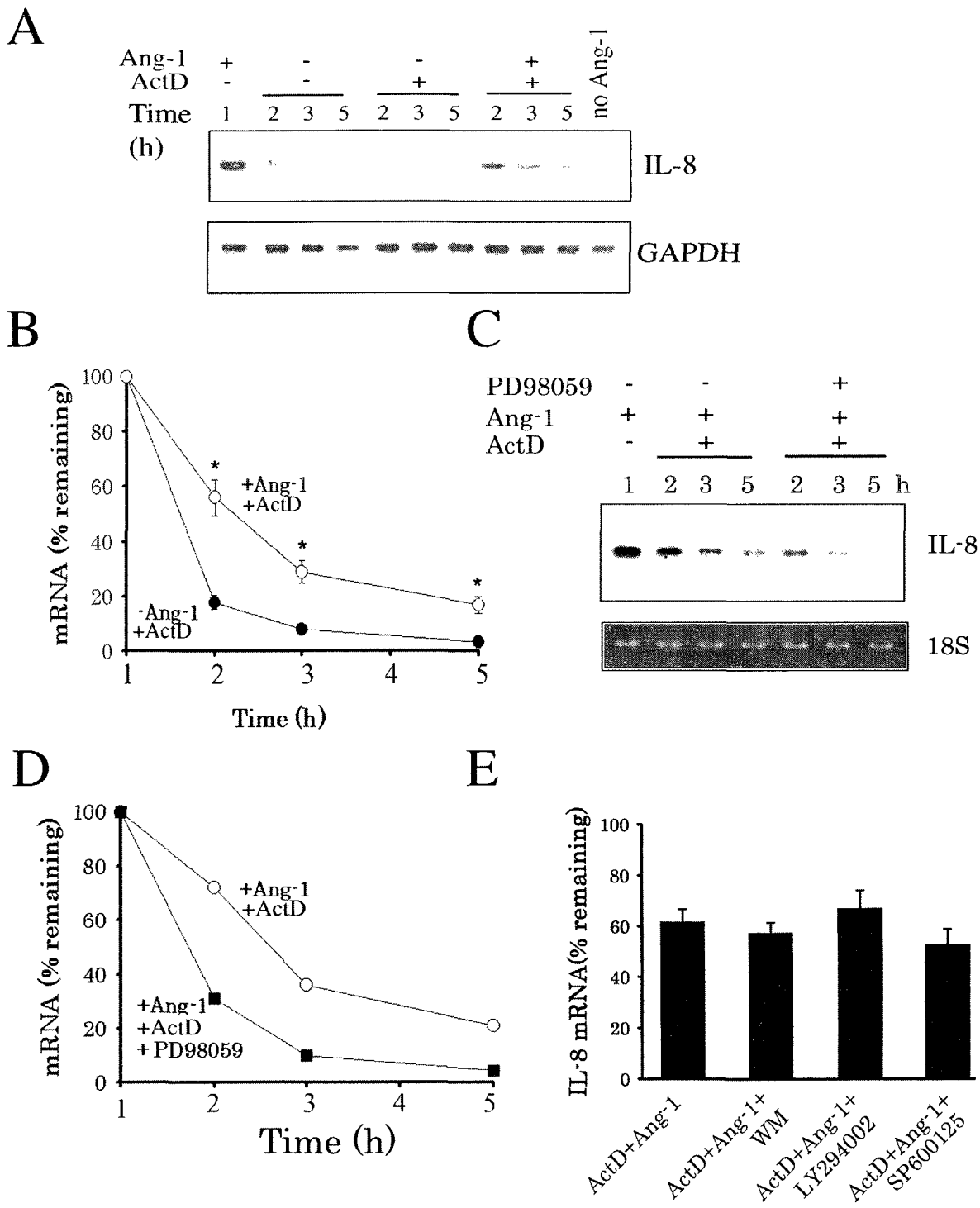

A
Ang-1
ActD
Time (h)
1 2 3 5 2 3 5 2 3 5
no Ang-1
IL-8
GAPDH
B
mRNA (% remaining)
+Ang-1 +ActD
-Ang-1 +ActD
Time (h)
C
PD98059
Ang-1
ActD
1 2 3 5 2 3 5 h
IL-8
18S
D
mRNA (% remaining)
+Ang-1 +ActD
+Ang-1 +ActD +PD98059
Time (h)
E
IL-8 mRNA(% remaining)
ActD+Ang-1
ActD+Ang-1+ WM
ActD+Ang-1+ LY294002
ActD+Ang-1+ SP600125

FIGURE 3.6

Figure 3.7: Roles of the transcription factor AP-1 in Ang-1-induced IL-8 production.

A: Top: structure of human IL-8 promoter (-133/+44) identifying AP-1 and NFκB-like binding elements. Sequences in brackets delineate in small letters mutations in these binding elements. Bottom: IL-8 promoter activity (normalized luciferase activity) measured in response to vehicle, Ang-1 and Ang-1 plus PD98059 (30μM), WM (50 nM) and SP600125 (15 μM). $*P<0.05$ compared with vehicle. Error bars represent SEM.

B: Binding of nuclear extracts from vehicle- and Ang-1 treated HUVECs to IL-8 specific AP-1 DNA probe in the absence (-) and presence of antibodies to c-Fos, FosB, c-Jun, JunB and JunD. Competition with cold and mutated probes was performed as specificity control.

C: Effects of Ang-1 (300 ng/ml) on phosphorylation of c-Fos (Thr^{325}), c-Jun (Ser^{63} and Ser^{73}), total c-Fos, c-Jun and JunD proteins.

D: The effect of 1 hour pre-treatment with PD98059 (30 μM), SP600125 (15 μM) and WM (50 nM) on c-Jun phosphorylation (Ser^{63} and Ser^{73}) measured after 15 minutes of Ang-1 (300 ng/ml) exposure.

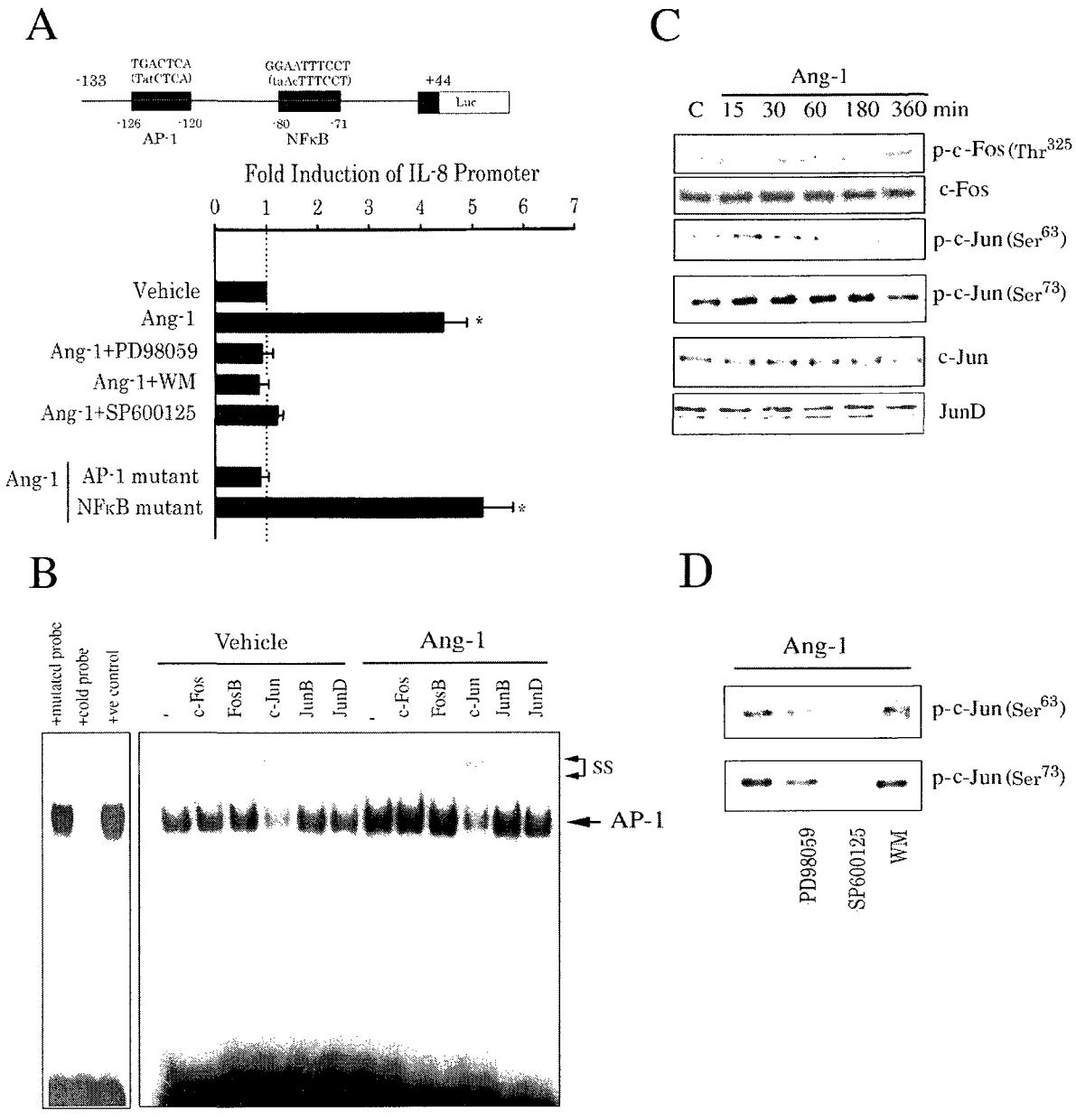

A
TGACTCA
(TatCTCA)
GGAATTTCCT
(taAcTTTCCT)
-133
+44
Luc
-126
-120
-80
-71
AP-1
NFκB
Fold Induction of IL-8 Promoter
0 1 2 3 4 5 6 7
Vehicle
Ang-1
Ang-1+PD98059
Ang-1+WM
Ang-1+SP600125
Ang-1
AP-1 mutant
NFκB mutant
C
Ang-1
C 15 30 60 180 360 min
p-c-Fos (Thr325)
c-Fos
p-c-Jun (Ser63)
p-c-Jun (Ser73)
c-Jun
JunD
B
+mutated probe
+cold probe
+ve control
Vehicle
Ang-1
- c-Fos FosB c-Jun JunB JunD - c-Fos FosB c-Jun JunB JunD
SS
AP-1
D
Ang-1
p-c-Jun (Ser63)
p-c-Jun (Ser73)
PD98059
SP600125
WM

FIGURE 3.7

Figure 3.8: A schematic depicting the signaling pathways through which the Ang-1/Tie-2 receptor pathway regulates IL-8 production in HUVECs.

Exposure to Ang-1 activates Tie-2 receptors, which in turn, provoke activation of the p38, Erk1/2, SAPK/JNK, and the PI-3 kinase pathways. While the Erk1/2 and SAPK/JNK pathways promote c-Jun phosphorylation and AP-1 activation leading to IL-8 induction, the mechanisms through which the PI-3 kinase pathway modulates IL-8 induction in response to Ang-1 are unclear, since this pathway didn't influence c-Jun phosphorylation. Also illustrated is the negative effect of the p38 MAPK pathway on Erk1/2 phosphorylation.

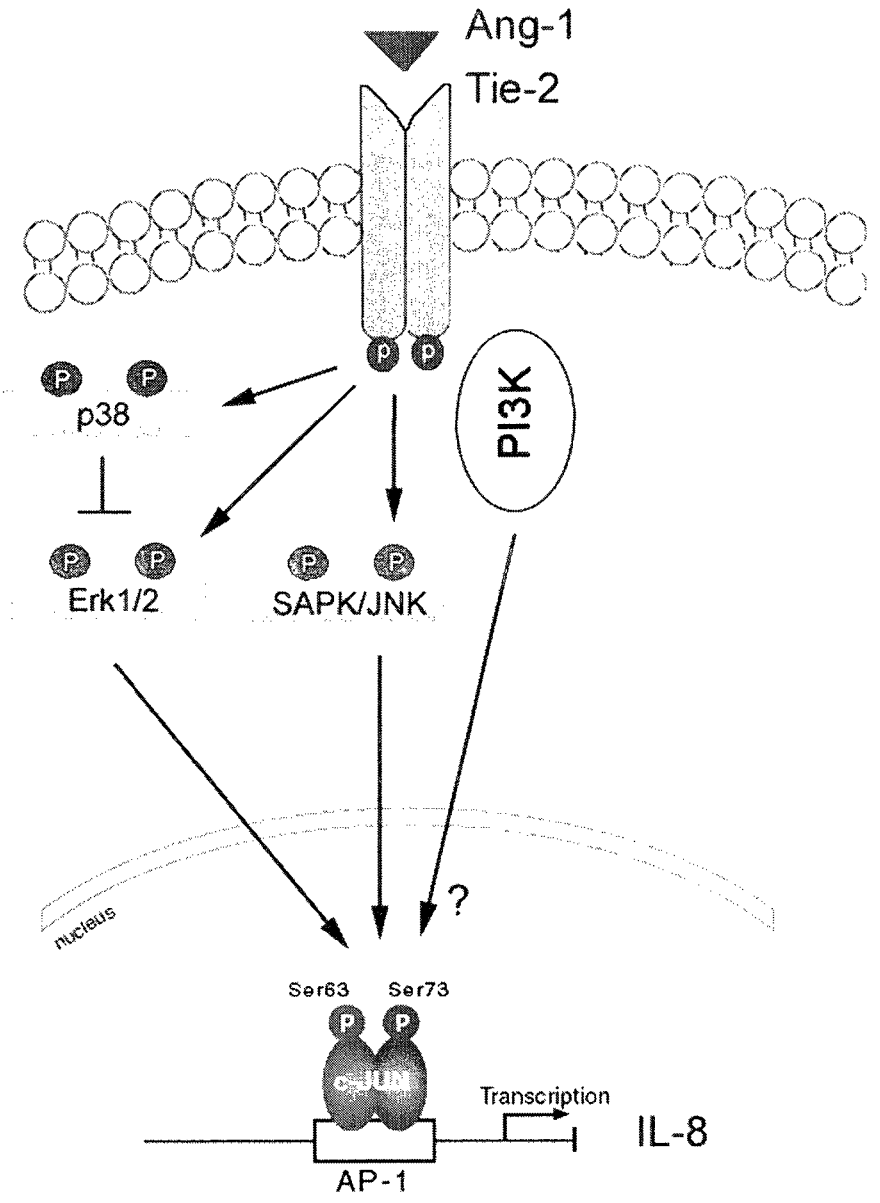

Ang-1
Tie-2
P
P
p38
PI3K
Erk1/2
SAPK/JNK
nucleus
?
Ser63
Ser73
c-JUN
Transcription
IL-8
AP-1

FIGURE 3.8

3.8. SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS:

Materials:

Most cell culture reagents were obtained from Invitrogen Corporation (Burlington, ON). The endothelial cell growth supplement (ECGS) was supplied by Biomedical Technologies Inc. (Stoughton, MA). Recombinant human Ang-1 protein and its cross-linking antibody, the IL-8 ELISA kit and the monoclonal anti-human IL-8 neutralizing antibody were purchased from R&D systems (Minneapolis, MN). The control mouse IgG and antibodies selective to total p50 and total p65 NFkB subunits and the antibodies used for the gel supershift experiments were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Commercial antibodies for Tie-1, Tie-2, total and phospho Erk1/2, phospho and total p38α, phospho and total p65 NFκB (Ser536), total IκBa and cleaved and total caspase-3 were purchased from Alpha Diagnostic International (San Antonio, TX), Calbiochem (San Diego, CA), Cell Signaling Technology (Danvers, MA), and BD Biosciences (Mississauga, ON). Antibodies for phospho-c-Jun (Ser^{63} and Ser^{73}) and phospho-c-Fos (Thr^{325}) were obtained from Cell Signaling and Invitrogen (Burlington, ON), respectively. RNeasy Mini Kit for total RNA isolation was supplied by Qiagen Inc. (Mississauga, ON). The pharmacological inhibitors PD98059, SB203580 and PD169316 were purchased from Calbiochem (San Diego, CA), U01256 from New England Biolabs and cycloheximide, actinomycin D, wortmannin and LY294002 from Sigma (St Louis, MO). Plasmids encoding the Flag-tagged wild-type p38α (pCMV-Flag-p38α) were generously provided by Roger J. Davis (University of Massachusetts Medical School, Worcester, MA). Adenoviruses expressing GFP (Ad-GFP), a dominant negative mutant form (K44M) of IKKα(Ad-dnIKKα) or a dominant negative mutant form (K44A) of IKKβ (Ad-dnIKKβ) were purchased from the Gene Transfer Vector Core of the University of Iowa (Iowa City, IA). The Gelshift NFκB/Rel family kit was obtained from Active Motif (Carlsbad, CA). The T4 polynucleotide kinase was purchased from Fermentas Life Sciences (Burlington, ON). The HUVEC Nucleofector Kit for plasmid transfections was obtained from Amaxa Inc (Gaithersburg, MD).

Cell culture

HUVECs were purchased from GlycoTech Corporation (Gaithersburg, MD). They were cultured in endothelial basal medium (MCDB131) supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement, 2mM glutamine, heparin, penicillin (100 units/ml), streptomycin (100 mg/ml) and amphotericin B (0.25 mg/ml). After reaching the first confluence in 100 mm dishes, the cells were sub-cultured following trypsin (0.025%) and EDTA (0.01%) application and plated at a density of 2500 cells/cm^2. Cells were used between passages 2 and 5.

Generation of the MSCV-ctrl and MSCV-Ang1 HUVECs

HUVEC-MSCV and HUVEC-MSCV-Ang1 were constructed by retroviral transduction using MSCV-pac (empty vector) or MSCV-pac containing murine Ang-1 cDNA, respectively. Transient transfections of the Ampho Phoenix packaging cell line were performed using Fugene 6 transfection reagent (Boehringer Mannheim, Laval, Quebec). Viral supernatants from transfected packaging cell lines were used to transduce the ECs in multiple rounds of infection. HUVECs were selected in puromycin. Expression of Ang-1 was confirmed by immunoblotting, using an antibody against the v5 tag and by real-time PCR, using mouse Ang-1 primers.

Northern blot analysis of IL-8 mRNA

HUVECs were serum starved for 12 h prior to the treatment with or without Ang-1 (300 ng/ml). In few experiments, cells were pretreated for 1 h with pharmacological inhibitors of MAPKs and the PI-3 kinase pathways. Cells were then lysed and total RNAs were isolated using the RNeasy Mini Kit from Qiagen Inc. (Mississauga, ON) according to the manufacturer's instructions. 5 mg of total RNA were loaded on a 1% formaldehyde-agarose gel. After electrophoresis, the RNAs were transferred overnight on a positively charged nylon membrane by capillary transfer. Cross-linking of the RNA to the membrane was performed by baking for 1 h at 80°C using a vacuum oven. The non-isotopically labeled IL-8 DNA probe were designed and labeled in our laboratory using RT-PCR and the PCR Digoxigenin probe synthesis kit supplied from Roche Diagnostics (Laval, QC). Based on the IL-8 mRNA sequence ID (NM_000584), the IL-8 probe was

generated to be complementary to the sequence spanning 5' to 3' from base 47 to base 344. 18s rRNA ethidium bromide staining was used as a loading control. The pre-hybridization (1 h) and the hybridization (overnight) of the membrane were performed at 42°C in the ULTRAhyb buffer from Ambion (Austin, TX). The membrane was then washed twice 5 min at room temperature with the low stringency wash solution (2x SSC/0.1%SDS) and twice 15 min at 42°C with the high stringency wash solution (2x SSC/0.1%SDS). The signal was detected using the DIG Luminescent detection kit and the CPD-Star, ready to use supplied by Roche Diagnostics. The membrane was then exposed to BioMax Light film purchased from Amersham Biosciences (Piscataway, NJ). The blots were scanned with an imaging densitometer (model GS700, 12-bit precision and 42- mm resolution, Bio-Rad, Hercules, CA), and optical densities of the bands were quantified with ImagePro Plus software (MediaCybernetics, San Diego, CA).

Measurement of secreted IL-8 by ELISA

HUVECs were serum starved for 4 h, then were either pretreated or not for 1 h with the soluble Tie-2 receptors, cycloheximide, or the pharmacological inhibitors of Erk1/2, SAPK/JNK, and PI-3 kinase pathways. Cells were then stimulated with Ang-1 (300 ng/ml) or Ang-1 solvent (0.1 % BSA in PBS) for 12 h. In few experiments, HUVECs were infected overnight with adenoviruses, allowed to recover in complete media for 24 h, then serum starved and treated with Ang-1 as mentioned above. The levels of secreted IL-8 in the culture supernatants were measured using a commercial IL8-ELISA kit from R&D systems.

Real-time PCR

This technique was used to evaluate mRNA expression of Ang-1 and Ang-2 in MSCV or MSCV-Ang1 HUVECs and to measure mRNA expression of Jun and Fos family members in response to 1 h of Ang-1 (300 ng/ml) exposure in regular HUVECs. MSCV and MSCV-Ang-1 HUVECs cells were grown to ~ 80-90 % confluence then they were serum starved for 8 h, harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Similarly, serum starved HUVECs were exposed for 1 h to vehicle or Ang-1 (300 ng/ml) and RNA was extracted

as described above. Quantification and purity of total RNA was assessed by A260/A280 absorption. 2 μg of RNA were reverse transcribed using the superScript II RNase H-Reverse transcriptase enzyme from Invitrogen Inc. Reactions were incubated at 42 °C for 50 min and at 90 °C for 5 min. Real-time PCR was performed using Prism 7000 Sequence Detection System from Applied Biosystems Inc. (ABI, Foster City, CA). Mouse Ang-1 and human Ang-2 Taqman assays were purchased from ABI. Briefly, 10 μl of the Taqman 2x PCR Master Mix (ABI) was added to a premix containing 1.5 ml (100 ng) of the reverse transcriptase reaction, 1ml of the Taqman probe (mouse Ang-1 or human Ang-2) and 7.5 ml of RNase and DNase-free distilled water. The thermal profile was as follows: 95 °C for 10 min, and 50x (95 °C for 15 s, 60°C for 1 min). All real-time PCRs were performed in triplicate. To determine the copy number of the mRNA in our samples, we established standard curves relating the CT (threshold cycle) values to the copy numbers. These curves were generated by plasmids containing full coding sequences of mouse Ang-1 or human Ang-2 (as per ABI Applied Biosystems instructions). For AP-1 family members, we designed specific primers (see Table 1) for c-Jun, JunB, JunD, c-Fos, FosB, Fra1, Fra2 and GAPDH (control). One μl of the reverse transcriptase reaction was added to 25 μl of SYBR Green PCR Master Mix (ABI) and 3.5 μl of each 10μM primer. The thermal profile was as follows: 95 °C for 15 min, and 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72°C for 33 s. A melt analysis for each PCR experiment was used to assess primer-dimer formation or contamination. A single melt peak observed for each set of primers was used to validate that only a single PCR reaction was generated. Results were analyzed using the comparative threshold cycle (CT, the value where the amplification curve crosses the threshold line) method where:

DCT=CT of gene of interest - CT of GAPDH

DDCT= DCT of Ang-1 treated- DCT of vehicle treated

Relative expression at a given time point was calculated as 2-DDCT. All Real-time PCR experiments were performed in triplicate.

Immunoblotting

Immunoblotting was used to detect MAPK phosphorylation, expression of AP-1 subunits, activation of caspase-3, activation of the NFκB pathway, expression of Ang-1,

Tie-1 and Tie-2 by MSCV-Ang1 cells and over-expression of p38a in pCMV- Flag-p38a transfected HUVECs. Total cell lysates (40-80 μg total protein) were boiled for 5 min and then loaded onto tris-glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked with 5% non-fat dry milk, and subsequently incubated with the specific antibodies. Activation of caspase-3 was determined by using antibodies specific to the cleaved forms of caspase-3. Activation of Erk1/2 was assessed with polyclonal antibodies specific to active (dually phosphorylated at Thr^{202} and Tyr^{204}) and total Erk1/2. Similarly, SAPK/JNK activation was evaluated using polyclonal antibodies specific to active (dually phosphorylated at Thr^{183} and Tyr^{185}) and total SAPK/JNK proteins. Proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagents (Chemicon, Temecula, CA). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the Bio-Rad protein assay with BSA as a standard (Bio-Rad Laboratories, Canada, Mississauga, ON).

mRNA stability Assay

After treatment with Ang-1 (300 ng/ml) for 1 h, cells were washed with PBS and were then maintained in serum starvation medium, medium containing actinomycin D (5 mg/ml) and medium containing Ang-1 plus actinomycin D. To evaluate the involvement of Erk1/2, PI-3 kinase and SAPK/JNK pathways in the regulation of IL-8 mRNA stability in response to Ang-1, additional groups of cells were maintained following in medium containing Ang-1 plus actinomycin plus PD98059, wortmannin, LY294002 or SP600125. RNA was then extracted and northern blot analysis for IL-8 mRNA was performed.

Cytosolic and nuclear extracts preparation

Cytosolic and nuclear extracts were obtained from cells grown to 85% confluence in 100-mm^2 dishes and treated with Ang-1 (300 ng/ml) for 30 min or 1 h. The extraction was performed on ice. Cells were harvested by scraping and were initially lysed with 400 ml of ice-cold hypotonic buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1mM Na_3VO_4, 5

μg/ml aprotinin, 1 μg/ml pepstatin, and 2 μg/ml leupeptin). After 15 min on ice, nonidet P-40 was added to a final concentration of 0.6% (v/v), and nuclei were centrifuged at 14000 rpm for 5 min. Supernatants containing cytoplasmic proteins were stored at -70 °C. The pelleted nuclei were re-suspended in 50 ml of ice-cold high salt buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1mM Na3VO4, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 2 μg/ml leupeptin). The lysed nuclei were vigorously rocked for 15 min at 4 °C and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants containing nuclear proteins were aliquoted and stored at -70 °C until used for the gel shift assay.

IL-8 promoter activity

Analysis of human IL-8 promoter revealed that induction of IL-8 transcription in response to inflammatory cytokines is mediated through binding of NFκB and AP-1 proteins at specific sites residing within 133 upstream from the transcription initiation site [12]. We analyzed in this study a firefly luciferase reporter plasmids driven by the -133/+44 bp segment of human IL-8 promoter [12]. We also studies two additional constructs in which the AP-1 binding element (-126 to -120 bp) and NFκB-like factor binding element (-80 to -71 bp) from the IL-8 gene have been mutated [12]. HUVECs seeded in 24-well plates were transiently co-transfected with 0.025 mg of pRL-TK and 0.25 mg of one of the aforementioned firefly luciferase gene reporter constructs using the Lipofectamine LTX reagent (Invitrogen Inc.) according to the manufacturer's instructions. Cells were treated with either solvent or 300 ng/ml of Ang-1 for 8 h. Firefly luciferase activity was measured using the Dual Luciferase Assay kit (Promega Inc. Madison, WI) and normalized to the relative renilla luciferase activity. In some experiments, cells were pretreated for 1 h with PD98059, wortmannin and SP600125 prior to stimulation with Ang-1.

Gel shift Assay

The p65-NFκB oligo probes provided with the Gelshift NFκB/Rel family kit and double-stranded AP-1 oligo probes containing the AP-1 binding element on human IL-8 promoter (5'-GTG TGA TGA CTC AGG TTT G-3') sequence were end-labeled using

the [g-32P] ATP and T4-kinase according to the manufacturer's instructions. The nuclear extracts were prepared as described above. The binding reactions were conducted with 5 μg (for AP-1) or 10 μg (for NFκB) of nuclear extract according to the manufacturer's instructions. In competition assays, 20x unlabeled wild type and mutated competitors were added at the same time of probe addition. For probe competition analysis, we used the above-described wild type oligos and mutated AP-1/IL-8 oligos (5'-GTG TGA TGA CCC AGG TTT G-3'). For the supershift analysis, 2 mg of antibodies selective for c-Fos, FosB, c-Jun, JunB and JunD were added to the nuclear extract before addition of the labeled probe. The binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis through 5% gels in a tris-borate buffer system. The gels were then fixed, dried, and exposed to an autoradiography film at -80°C.

Transfection of HUVECs with DNA plasmids

HUVECs were transfected using the Amaxa nucleofection technology. Briefly, 1x106 cells were re-suspended in 100 ml of the HUVEC Nucleofector solution and mixed with 1 mg of pCMV-Flag-p38a. Then, the mixture of cells and plasmid DNA was transferred to a provided cuvette and pulsed with the Amaxa Nucleofector I apparatus. Cells were then immediately transferred to wells of 6-well plate containing pre-warmed cell culture media and cultured for 48 h. Transfected cells were then lysed and proteins were extracted. Lysates were assayed by western Blotting to confirm the overexpression of the Flag-tagged p38α and to study the effect of p38α over-expression on Erk1/2 phosphorylation.

Preparation of secreted and matrix-associated Ang-1 protein produced by MSCV-Ang1 cells

The cell culture supernatants of HUVEC-MSCV and HUVEC-MSCV-Ang1 were collected and their protein contents were concentrated using the acetone precipitation method. The cells layers were removed from the culture dishes by treatment with 5mM EDTA in PBS. The extracellular matrix components left behind on the culture dishes were extracted with the SDS Laemmli sample buffer containing 15% β-mercaptoethanol. Samples were analyzed by immunoblotting. To quantify Ang-1 content of the samples,

known amounts of recombinant human Ang-1 were run in parallel to generate a standard curve (OD versus amount of Ang-1).

Adenoviral infection of HUVECs

HUVECs (60-70 % confluent) were infected overnight at a multiplicity of infection of 50 in serum-free medium. The virus-containing medium was then replaced with complete medium containing 20% FBS and endothelial cell growth supplement for 24 h. The cells were then used to perform further experiments.

Proliferation Assay

We assessed cell proliferation using two different assays: cell count and the BrdU incorporation. For the cell count, MSCV and MSCV-Ang1 cells were plated in 12-well plates at a density of 7 x 10^4 cells/well in MCDB131 medium containing 2% FBS. After 2 days, cells were trypsinized and counted using a hemacytometer. In few experiments, cells were treated with either monoclonal anti-IL8 neutralizing antibody (7.5 μg/ml) or with normal mouse IgG (7.5 μg/ml) as a control. For the BrdU incorporation, we used the Cell Proliferation ELISA BrdU colorimetric kit from Roche Applied Science (Laval, Quebec). MSCV and MSCV-Ang1 cells were plated in 96-well plates at a density of $5x10^3$ cells/well in 100 ml of MCDB131 containing 2% FBS. The next day, cells were pulsed with 10 mM of BrdU and incubated for an additional 24 h. Cells were then fixed and labeled according to the manufacturer's instructions. The absorbance of the samples was then measured in a microplate reader at 370 nm 10 min after the addition of the substrate. As for the cell count, in some experiments cells were treated at the time of plating with either monoclonal anti-IL8 neutralizing antibody or with normal mouse IgG.

Validation of IL-8 neutralization with anti-IL-8 antibody

To validate that neutralizing anti-IL-8 antibody at 7.5 μg/ml is capable of blocking the effects of IL-8 on EC proliferation, we measured the influence of this antibody on recombinant IL-8-induced proliferation of HUVECs. HUVECs were seeded ($5x10^3$ cells) in 96-well plates and were then divided into three groups. Group 1 was untreated whereas group 2 was treated with 10 ng/ml of recombinant human IL-8. Group

3 was treated with recombinant IL-8 protein and monoclonal anti-IL-8 neutralizing antibody (7.5 μg/ml). The three groups of cells were cultured in MCDB131 medium containing 2% FBS for 24 h. Cells were then pulsed with 10 mM of BrdU and incubated for an additional 24 h. Cells were then fixed and labeled according to the manufacturer's instructions and then BrdU absorbance was measured as described.

Cleaved caspase-3 intensity

MSCV and MSCV-Ang1 cells were plated in MCDB131 medium containing 2% FBS. After 2 days, cells were lysed and proteins were extracted. Fifty mg of proteins were loaded on an SDS- polyacrylamide gel and subjected to western Blotting. Membranes were probed with a polyclonal antibody specific for cleaved caspase-3 (Cell Signaling Inc.). In few experiments, at the time of seeding, cells were treated with either monoclonal anti-IL8 neutralizing antibody or with normal mouse IgG as a control.

Wound healing assay

Cell motility was assessed using the wound-healing assay. MSCV or MSCV-Ang1 cells were grown for 24 h in basal medium. The media was collected and incubated at room temperature with anti-IL8 neutralizing antibody (7.5 μg/ml) or IgG control antibody (7.5 μg/ml). Fresh MSCV and MSCV-Ang-1 were seeded into 24-well tissue culture plates and cultured in complete medium containing 20% FBS to nearly confluent cell monolayers. The cells were then carefully wounded using a 200 μl pipette tip. Cellular debris was removed by washing with PBS. After making wounds, the culture medium was switched to the conditioned media of MSCV and MSCV-Ang-1 neutralized with anti-IL-8 or control IgG antibody. Wounds were then photographed (time = 0) and 12 h later. Migration was evaluated by measuring the reduction in the diameter of the wound after migration of the cells into the cell-free zone. The experiments were repeated in triplicate wells twice.

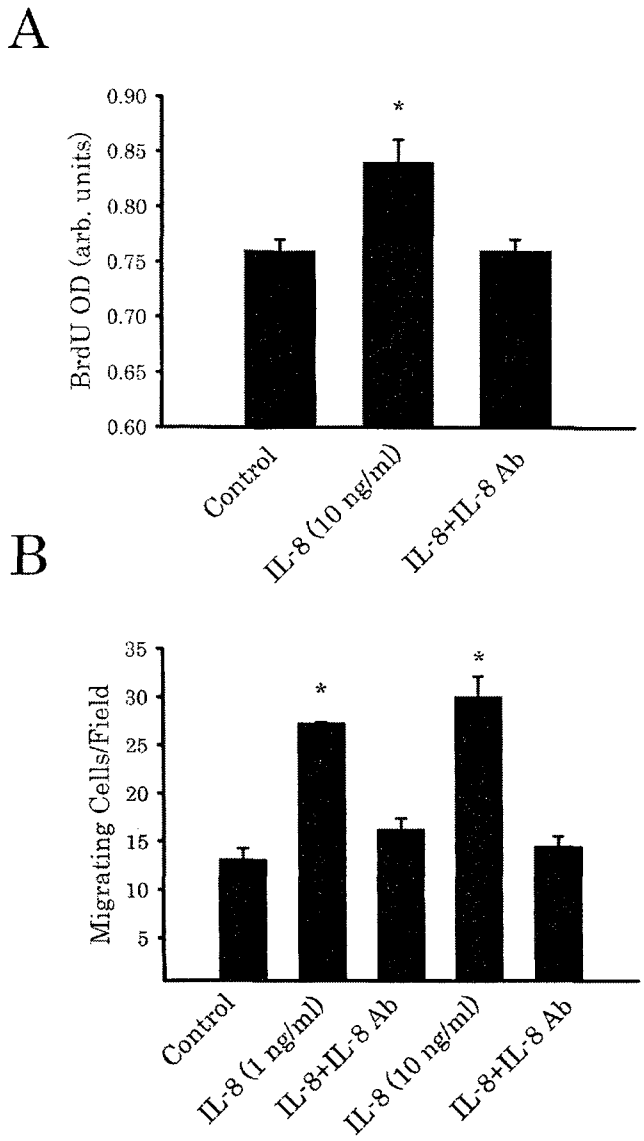
Migration assay

EC migration was performed in 24-well transwell polycarbonate inserts with a pore size of 8.0 mm (Corning, Life Sciences, Acton, MA). The inserts were coated with

50-mg/ml fibronectin for 2 h at 37°C. Sub-confluent normal HUVECs (not transduced by retroviruses) were washed, trypsinized, and resuspended at 10^5 cells/100 µl in basal medium and seeded in the upper compartment. MSCV or MSCV-Ang-1 cells were grown in basal media for 24 h and media were then collected and incubated with anti-IL-8 and control IgG antibodies as described above. Conditioned media were then placed into the lower compartment of the migration apparatus. The apparatus was then incubated at 37°C in a CO_2 incubator for 5 h to allow for cell migration. The inserts were then fixed with 10% buffered formalin for 20 min and stained with Giemsa solution for 30 min. The upper side of the insert was subsequently scraped with cotton swabs to remove non-migrating cells. Migration was quantified using ImagePro Plus software by counting cells in 10 fields (×200) per well. For verification of IL-8 neutralization with a specific antibody, normal HUVECs ($7.5x10^4$ cells/100µl) were seeded in the upper chamber in basal medium. The lower chamber contained 500 µl of basal medium containing control IgG (control condition), recombinant human IL-8 (1 and 10 ng/ml) and recombinant IL-8 plus anti-IL-8 antibody (7.5 µg/ml). Migration was measured 5 h later as described above.

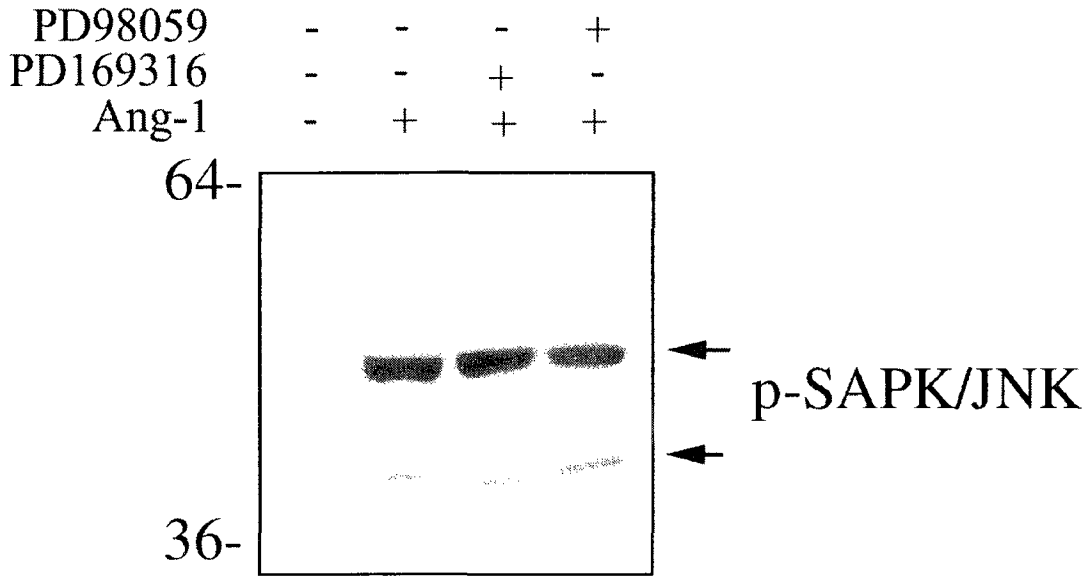
Table ST3. 1: Primer used for real-time PCR experiments.

Type	Sequence (5' to 3')		Accession #	expecte size (Bp)
c-Jun	Fwd	ACGGCCAACATGCTCAGG	NM_002228	101
	Rev	TGTTTGCAACTGCTGCGTTAG		
JunB	Fwd	GAGCTCGTACCCGACGACCAC	NM_002229	218
	Rev	TTCCGCAGCCGCTTGCGCTCCAC		
JunD	Fwd	GCCGCCTCCAAGTGCCGCAAG	NM_005354	208
	Rev	CGGACTCAGTACGCGGGCACCTG		
c-Fos	Fwd	GAAGACCGAGCCCTTTGATG	NM_005252	200
	Rev	TACAGGTGACCACCGGAGTG		
FosB	Fwd	AGGCATGAGTGGCTACAGCA	NM_006732	284
	Rev	CGATCTCCGACTCCAGCTCT		
Fra-1	Fwd	GTCAGGAGCTGCAGTGGATG	NM_005438	167
	Rev	CGATCTCCGACTCCAGCTCT		
Fra-2	Fwd	GCTCAGGCAGTGCATTCATC	NM_005253	100
	Rev	GGGTTGGACATGGAGGTGAT		
GAPDH	Fwd	AAGAAGGTGGTGAAGCAGGCG		166
	Rev	ACCAGGAAATGAGCTTGACAA		



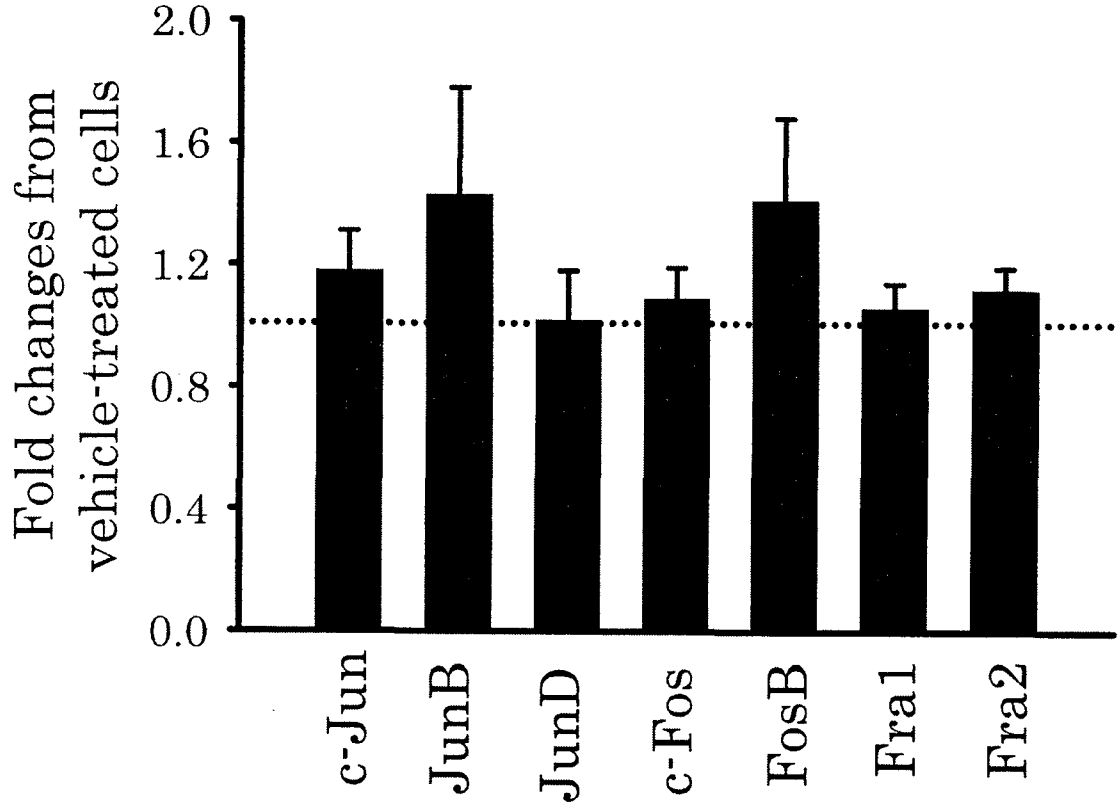
Supplementary Figure S3.1: Effects of anti-IL-8 antibody on IL-8-induced proliferation and migration of HUVECs.

For BrdU measurements, cells were exposed to recombinant IL-8 for a total of 48h. For migration assay, IL-8 (with or without neutralizing antibody) was placed into the lower chamber and migration was measured after 5 h. *P<0.05 compared with control.



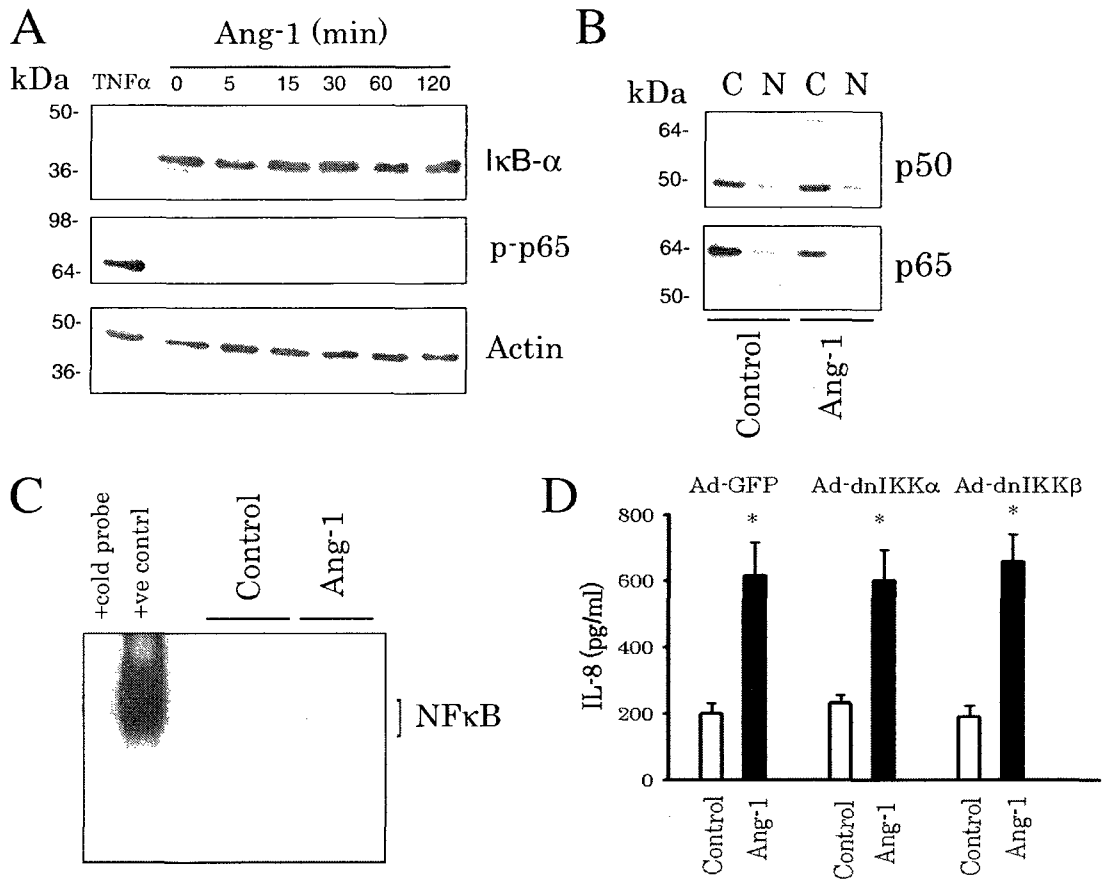
Supplementary Figure S3.2: Effects of inhibition of Erk1/2 (30 mM PD98059) and p38 (10 mM PD169316) MAPKs on Ang-1-induced phosphorylation of SAPK/JNK proteins (Thr^{183}/Tyr^{185}).

Note that inhibition of Erk1/2 and p38 MAPK pathways had no effect on Ang-1-induced SAPK/JNK phosphorylation.



Supplementary Figure S3.3: Changes in mRNA expression of c-Jun, JunB, JunD, c-Fos, FosB, Fra1 and Fra2 measured after treatment of HUVECs with Ang-1.

Changes in mRNA expression (measured with real-time PCR) of c-Jun, JunB, JunD, c-Fos, FosB, Fra1 and Fra2 measured after 1 h of treatment of HUVECs with 300 ng/ml of Ang-1. Data are means± SEM (n=6) and are expressed as fold changes from HUVECs treated with Ang-1 vehicle. Note that Ang-1 exposure had no significant influence on the expression of these proteins.



Supplementary Figure S3.4: Ang-1 does not activate the transcription factor NFκB.

A: Changes in total IκB-α and p65 NFκB phosphorylation (Ser^{536}) in response to Ang-1. Actin used as loading control and TNF-α treatment (10 min exposure) as positive control.

B: Mobilization of p50 and p65 NFκB subunits in response to Ang-1 treatment. Cytosolic (C) and nuclear (N) fractions were analyzed with immunoblotting.

C: Binding of nuclear extracts from Ang-1 treated HUVECs to specific NFκB DNA probes. Nuclear extracts provided by kit were used as positive control. Competition with the cold probe performed as specificity control.

D: HUVECs transfected with adenoviruses expressing GFP, dominant negative IKK-α or IKK-β proteins. Cells then exposed for 12h to Ang-1, IL-8 protein then detected in culture media. Data are expressed as mean± SEM (n=6).

RATIONALE FOR CHAPTER 4

The transcription factors that are activated downstream from the Ang-1/Tie-2 receptor system and that modulate gene expression promoting angiogenesis are unclear. In chapter 2, we identified the immediate early gene Egr-1 as one of few transcription factors that was significantly upregulated downstream from the Ang-1/Tie-2 receptor pathway. A recent study implicated Egr-1 in mediating the pro-angiogenic effects of the growth factor FGF-2 in ECs. Therefore, we hypothesized that Egr-1 is induced in response to Ang-1 in ECs and that this transcription factor contributes significantly to the regulation of proliferation and migration of these cells downstream from Tie-2 receptors.

CHAPTER 4

Article Type: Original Contribution

EARLY GROWTH RESPONSE-1 REGULATES ANGIOPOIETIN-1-INDUCED ENDOTHELIAL CELL PROLIFERATION AND MIGRATION

Nelly A. Abdel-Malak[1], Dominique Mayaki[1], Levon M. Khachigian[2] and Sabah N.A. Hussain[1]

[1]Critical Care Division, Department of Medicine, McGill University Health Centre, McGill University, Montreal, Québec, Canada.

[2]Centre for Vascular Research, University of New South Wales, Sydney, Australia.

Short Title: Contribution of Egr-1 to angiopoietin-1 signaling

Address of Correspondence: Dr. S. Hussain,
Royal Victoria Hospital,
Room L3.05,
687 Pine Ave West,
Montréal, Québec, Canada H3A 1A1
Tel. (514) 843-1664
Fax (514) 843-1686

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4.1. ABSTRACT

Objective: It is well known that the angiopoietin-1 (Ang-1) inhibits apoptosis and promotes migration and proliferation of ECs. However, the transcription factors involved in these effects remain unclear. In this study, we evaluated the contribution of early growth response-1 (Egr-1) to Ang-1-induced migration and proliferation in HUVECs.

Methods: Expression of Egr-1 was evaluated with real-time PCR and immunoblotting, whereas Egr-1 DNA binding activity was monitored with electrophoretic mobility shift assays. Cell migration was measured with Boyden chambers and wound healing assays, whereas cell proliferation was monitored with cell counting and BrdU incorporation. To selectively inhibit Egr-1 expression, we used both siRNA oligonucleotides and specific DNAzymes.

Results: Egr-1 mRNA expression rose by about 9-fold within 2h of Ang-1 exposure and declined thereafter. Upregulation of Egr-1 expression was accompanied by increased nuclear mobilization and augmented DNA binding and was mediated through the Erk1/2, PI-3 kinase and mTOR pathways. Knocking down Egr-1 expression completely abrogated Ang-1-induced endothelial cell migration and significantly reduced proliferation of HUVECs over-expressing Ang-1.

Conclusion: Ang-1 exposure triggers, in ECs, a significant and transient induction of Egr-1 and this transcription factor contributes to Ang-1-induced migration and proliferation of these cells.

Key words: angiogenesis, Ang-1, Egr-1, proliferation, migration

4.2. INTRODUCTION

The receptor tyrosine kinase (Tie-2) and its ligands, the angiopoietins (angiopoietin-1,-2,-3 and -4), have emerged as important regulators of angiogenesis both in adults and in embryos (1;2). In adult mice, Ang-1 stimulates *in vivo* vascular remodelling, vascular enlargement, wound healing and lymphangiogenesis (3-5). In addition, Ang-1 inhibits endothelial cell (EC) apoptosis (6;7) stimulates migration, sprouting and differentiation of these cells (8;9).

Despite its importance in vascular homeostasis and angiogenesis, little is known about the transcription factors that are activated downstream from the Ang-1/Tie-2 receptor pathway. Daly *et al.* (10) have reported that Ang-1 inhibits the transcriptional activity of FoxO-1 (FKHR) transcription factor, thereby, reducing the expression of several pro-apoptotic proteins. Another transcription factor activated downstream from the Erk1/2 pathway by Ang-1 is Elk-1, which forms a complex by binding serum response factor and serum response element in various promoters (11). Finally, NERF2, an Ets family transcription factor, has been shown to be induced in ECs in response to Ang-1 exposure (12). It should be emphasized that in none of the studies did the authors address the importance of the above-described transcription factors in mediating the biological functions of the Ang-1/Tie-2 pathways.

We have recently profiled the transcriptome of EC exposed to Ang-1 using microarray technology and identified the early growth response-1 (Egr-1) as one of few transcription factors that was significantly induced downstream from Tie-2 receptors (13). Egr-1 is an immediate-early gene that is rapidly and transiently induced by many stimuli, including hypoxia, shear stress and injury and once activated, it binds promoter regions of several growth factors, cytokines, receptors and adhesion molecules (14). Two co-repressors of Egr-1, Nab-1 (constitutive) and Nab-2 (inducible) negatively regulate the activity of Egr-1 through direct interaction (15). Moreover, Egr-1 knockdown or over-expression of Nab-2 attenuates proliferation and differentiation of ECs evoked by fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) (16;17). No study has thus far linked Egr-1 to Ang-1/Tie-2 signaling in ECs. The main aim of this study is to test the hypothesis that Egr-1 is induced downstream from the Ang-1/Tie-2 receptor pathway in ECs and that this transcription factor contributes

significantly to the regulation of EC proliferation and migration by the Ang-1/Tie-2 receptor pathway.

4.3. MATERIALS AND METHODS

Cell culture

HUVECs were cultured in endothelial basal medium (MCDB131) supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplements, 2mM glutamine, heparin, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Cells were serum-starved overnight and were then stimulated with phosphate buffered saline (PBS, control) or 300 ng/ml of Comp-Ang-1, Ang-2 or Ang-4 proteins. COMP-Ang-1 is a soluble, stable and potent Ang-1 recombinant chimera made by replacing the amino terminal of Ang-1 with the short coiled-coil domain of cartilage oligomeric matrix protein (COMP) (18). Cells were collected 30 min, 1, 2, 4 and 6h later and examined for Egr-1 and Nab-2 mRNA expressions and Egr-1 protein expression. In addition, cells were exposed for 2h to VEGF-A (80 ng/ml), COMP-Ang-1 (300 ng/ml) and a combination of the two. To assess the nature of pathways regulating Egr-1 expression, serum-starved HUVECs were pre-incubated for 1h with U0126 (30 μM, inhibitor of Erk1/2 MAPKs), SB203580 (10 μM, inhibitor of p38 MAPKs), SP600125 (15 μM, inhibitor of SAPK/JNK pathway), wortmannin (50 nM, inhibitor of PI-3 kinase), rapamycin (50 ng/ml, inhibitor of mammalian target of rapamycin, mTOR) and a combination of U0126 and SB203580. Cells were then stimulated with COMP-Ang-1 (300 ng/ml) for 2h and RNA was extracted for Egr-1 expression.

Transfection with siRNA oligonucleotides

HUVECs were maintained in basal medium for 12h and were then transfected with 10 nM of either Egr-1-specific or scrambled negative control Dicer-substrate siRNA duplexes (Integrated DNA Technologies, Coralville, IA) using the HiPerfect transfection reagent (Qiagen, Mississauga, ON) following the manufacturer's instructions. Gene silencing was monitored after 24h by extracting the total RNA and performing real-time PCR experiments and immunoblotting with anti-Egr-1 antibody.

Transfection with DNAzymes: The RNA-cleaving phosphodiester-linked DNA-based enzymes (DNAzymes) are cation-dependent enzymes made up entirely of DNA that can be designed to cleave target mRNA in a gene-specific and catalytically efficient manner. DzF is a DNAzyme that was engineered to target the $A^{301}U$ site in the human Egr-1 mRNA (16). HUVECs were maintained starvation medium for 6h and were then transfected overnight with 0.05 μM of either DzF (Egr-1 DNAzyme) or Dz-Scr (scrambled DNAzyme) using the HiPerfect reagent. Gene silencing was monitored after 24h using real-time PCR.

Generation of the MSCV-ctrl and MSCV-Ang1 HUVECs

HUVECs were transduced with retroviruses expressing an empty MSCV-pac (empty vector) or MSCV-pac containing murine Ang-1 cDNA, respectively. Transient transfections of the Ampho Phoenix packaging cell line were performed using Fugene 6 transfection reagent (Boehringer Mannheim, Laval, Quebec). Viral supernatants from transfected packaging cell lines were used to transduce HUVECs in multiple rounds of infection and positively transduced cells were selected in puromycin.

Real-time PCR

Total RNA (2μg) was reverse transcribed using 200 Superscript II Reverse Transcriptase (Invitrogen) in a reaction mixture containing 0.5mM dATP, dCTP, dGTP, and dTTP, 40 units of RNase inhibitor, 50pmol random hexamers, 3mM $MgCl_2$, 75mM KCl, 50mM Tris-HCl (pH 8.3), and DTT 20mM in a total volume of 20μl. Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and primers designed to amplify human transcripts of Egr-1 and Nab2 (TaqMan® assays, Applied Biosystems) and VEGFR-1 (Flt-1), VEGF-A, VEGF-C, hepatocyte growth factor (HGF), heparin-binding EGF-like growth factor (HB-EGF), platelet-derived growth factor B (PDGF-B), FGF-2, the chemokine receptors CXCR4, interleukin-8 (IL-8) and GAPDH (Sybr Green assays, Table 1). These genes were detected after 24h of transfection of HUVECs with Egr-1 siRNA and scrambled siRNA oligonucleotides. We chose to detect these genes because they are known targets of the Ang-1/Tie-2 receptor pathway (VEGF-C, CXCR4, IL-8, Flt-1, HB-EGF and HGF) and

Egr-1-mediated transcription (PDGF-B, Flt-1, and FGF-2) (14;19-21). Results were analyzed using the comparative threshold cycle (C_T) as described in our recent study (19).

Electrophoretic mobility shift assay (EMSA)

The wild type Egr-1 oligonucleotide probes (5'-GGA TCC AGC GGG GGC GAG CGG GGG CGA-3') were end-labeled using the [γ-^{32}P] ATP and T4-kinase according to the manufacturer's instructions. The binding reactions of these probes with 5 μg of nuclear extracts of HUVECs stimulated with PBS or COMP-Ang-1 were performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA). In competition assays, 20x unlabeled wild type and mutated egr-1 oligos competitors (5'-GGA TCC AGC GGG TAC GAG CGG GTA CGA-3') were added at the same time of probe addition. For the supershift analysis, anti-Egr-1 antibody (2 μg) was added to the nuclear extract before addition of the labeled probe. The binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. The gels were then fixed, dried, and exposed to an autoradiography film.

Wound healing

Cells were maintained in complete medium to confluency and were then wounded using a 200 μl-pipette tip. PBS or COMP-Ang-1 (300ng/ml) was then added and wound healing was determined 12h later with an inverted bright field microscopy (Olympus Inc.) and was quantified by measuring the wounded area (Image Pro-Plus software) as described previously (22).

Migration assay

Migration of HUVECs was performed in 24-well trans-well polycarbonate inserts (8.0μm pore size) of modified Boyden chambers as described (23). HUVECs transfected with either Egr1-siRNA or scrambled-siRNA oligonucleotides were seeded in the upper compartment at a density of 10^5 cells/100 μl of basal medium. Ang-1 was then added into the lower compartment. The apparatus was then incubated at 37°C in a CO_2 incubator for 5h. Migration was quantified using ImagePro Plus software as described (23)

Proliferation assays

For cell counting, cells were cultured in MCDB131 medium plus 2% fetal bovine serum (FBS). After 48h, cells were trypsinized and counted using a hemacytometer. For BrdU incorporation, cells were plated in 96-well plates in MCDB131 containing 2%FBS. After 24h, cells were pulsed with 10 μM of BrdU and incubated for an additional 24h. Cells were then fixed and labeled according to the manufacturer's instructions (Roche Applied Science, Laval, QC). The absorbance of the samples was measured in a microplate reader at 370 nm.

Immunoblotting

Total cell lysates (40-80 μg total protein) were boiled for 5 min and then loaded onto tris-glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked with 5% non-fat dry milk, and subsequently incubated with the specific polyclonal antibodies. Activation of Erk1/2 was assessed with polyclonal antibodies specific to active Erk1/2 dually phosphorylated at Thr^{202} and Tyr^{204}. Proteins were detected with horseradish peroxidase-conjugated anti-goat or anti-rabbit secondary antibodies and ECL.

Data analysis

Data were expressed as means± standard errors (SE). Differences between experimental groups were determined by One Way Analysis of Variance and were considered statistically significant at $p<0.05$. A similar analysis was used to compare cells transfected with siRNA oligos and DNAzymes and between MSCV and MSCV-Ang-1 cells.

4.4. RESULTS

COMP-Ang-1 (300 ng/ml) elicited a significant induction of Egr-1 mRNA expression which peaked at 2h and declined thereafter to levels which were lower than control values (Figure 4.1A). Similarly, Ang-4 but not Ang-2 significantly induced Egr-1 expression measured after 2h of treatment (Figure 4.1B). VEGF (80 ng/ml) also induced

Egr-1 mRNA expression and when VEGF and COMP-Ang-1 were combined, the induction of Egr-1 mRNA was additive (Figure 4.1C). Inhibition of Erk1/2, PI-3 kinase and mTOR pathways significantly attenuated Ang-1-induced Egr-1 expression whereas SAPK/JNK inhibition with SP600125 had no effect (Figure 4.1D). Inhibition of the p38 MAPKs by SB203580 resulted in augmentation of Ang-1-induced Egr-1 expression, however, when SB203580 was present along with U0126, Egr-1 expression was attenuated to levels significantly lower than those observed with Ang-1 alone. These results suggest that the Erk1/2 and PI-3 kinase pathways promote whereas the p38 MAPK pathway inhibits Egr-1 expression and that the inhibitory effects of p38 MAPK on Egr-1 expression is mediated through a negative influence on Erk1/2 pathway as described in our study (24). To verify the inhibitory effect of p38 MAPKs on Erk1/2 phosphorylation, we pre-incubated HUVECs with two structurally unrelated inhibitors (SB203580 and PD169316 both at 10 μM) for 1h and cells were then stimulated with PBS (control) or 300 ng/ml of COMP-Ang-1 for 15 min. Inhibition of p38 significantly increased both basal and Ang-1-induced Erk1/2 phosphorylation thereby confirming the inhibitory effect of p38 MAPK on Erk1/2 phosphorylation (Figure 4.1E). In addition to Egr-1 induction, COMP-Ang-1 elicited within 30min a significant reduction in the expression of Nab-2 (negative regulator of Egr-1), however, expression of Nab-2 rose significantly an hour later with a return to control levels after 2h (Figure 4.1F). To confirm that regulation of Egr-1 mRNA expression was accompanied by similar regulation of Egr-1 protein, we performed immunoblotting of total cell lysates and cytosolic and nuclear fractions of HUVECs exposed to PBS or COMP-Ang-1. Figure 2 shows that COMP-Ang-1 elicited a significant and transient increase in total Egr-1 protein levels and an increase in nuclear accumulation of Egr-1 protein. Gel shift assays employing consensus Egr-1 DNA binding oligonucleotide sequence demonstrated that COMP-Ang-1 significantly enhanced Egr-1 DNA binding in a time-dependent manner (Figure 4.2C). The specificity of Egr-1 DNA binding was confirmed using supershift assays (Figure 4.2C).

To evaluate the involvement of Egr-1 in Ang-1-induced EC migration, we performed two assays, Boyden chamber migration and wound healing assays in which we used siRNA oligos and DNAzymes (DzF) to knockdown Egr-1 expression. Both

approaches significantly attenuated Egr-1 levels both in control and COMP-Ang-1-treated cells (Figure 4.3A). In cells transfected with scrambled DNAzymes (DzFSCR), COMP-Ang-1 significantly enhanced wound healing rates compared with control cells (Figure 4.3B). Transfection with Egr-1 DNAzyme (DzF) significantly reduced wound healing in HUVEC exposed to PBS (control) and COMP-Ang-1 (Figure 4.3B). Similarly, COMP-Ang-1 significantly increased migration of HUVECs transfected with scrambled siRNA oligos in the Boyden chamber assays compared with cells exposed to PBS (Figure 4.3C). Transfection with Egr-1 siRNA oligos reduced cell migration in both cells exposed to vehicle and to COMP-Ang-1 (Figure 4.3C).

To evaluate the involvement of Egr-1 in Ang-1-induced EC proliferation, we generated HUVECs that over-express Ang-1 (MSCV-Ang-1 cells) using a retroviral vector as described in our recent study (24). These cells produce and secrete into the culture medium Ang-1 protein, which gets incorporated into the extracellular matrix (Figure 4.4A). Both cell counting and BrdU incorporation assays confirmed that cells over-expressing Ang-1 (MSCV-Ang-1 HUVECs) proliferate faster than MSCV cells (Figure 4.4B). We also detected significantly greater levels of Egr-1 mRNA in MSCV-Ang-1 cells compared with control (MSCV) cells when these cells were maintained in the presence of 15% FSB and in the presence of basal medium alone (0%FBS) (Figure 4.4C). The higher rates of proliferation of MSCV-Ang-1 cells were not influenced by transfection with scrambled siRNA oligos or scrambled DNAzymes (Figure 4.4D&E). However, proliferation of MSCV-Ang-1 cells was significantly attenuated when Egr-1 expression was knocked down with siRNA oligos or DNAzymes (Figure 4.4 D&E). It should be noted that even in the presence of Egr-1 knockdown, proliferation of MSCV-Ang-1 cells remained higher than MSCV cells suggesting that Egr-1 plays a role but it is not only factor contributing to the increase in proliferation of HUVEC over-expressing Ang-1 (Figure 4.4D&E). To elucidate mechanisms through which Egr-1 promotes Ang-1-induced proliferation and migration, we studied the influence of knockdown of Egr-1 expression with siRNA oligos on the expression of specific pro-angiogenesis genes (Table 4.1). Our results indicate that transfection with Egr-1 siRNA oligos reduced the expressions of PDGF-B, FGF-2, IL-8 and Flt-1 but had no influence on VEGF-A, VEGF-C, HB-EGF, HGF and CXCR4 gene expressions (Table 4.1).

4.5. DISCUSSION

The main findings of this study include that: 1) Ang-1 induces in ECs through the Erk1/2, PI-3 kinase and mTOR pathways a significant increase in the expression and DNA binding of Egr-1; 2) The effects of Ang-1 and VEGF on Egr-1 mRNA expression are additive; 3) Egr-1 plays an important role in Ang-1-induced migration and proliferation of ECs; and 4) Inhibition of Egr-1 expression in ECs significantly attenuates the expression of selective pro-angiogenesis genes including PDGF-B, FGF-2, Flt-1 and IL-8.

Regulation of Egr-1 expression

In a recently published study, we described alterations in HUVEC transcriptome in response to Ang-1 exposure and reported that one of the highly induced transcription factors is Egr-1 (19). The current study explores both the mechanisms through which Ang-1 induces Egr-1 in HUVECs and the functional importance of this transcription factor in Ang-1-induced migration and proliferation of these cells. In vascular cells, Egr-1 is induced by many stimuli including hypoxia, cyclical strain, insulin, thrombin and several growth factors including VEGF-A, FGFs and EGFs (for review, see (25)). There is also evidence that induction of Egr-1 expression by these growth factors is highly dependent on the Erk1/2 pathway and involves Elk-1 and serum response factor (26). The Erk1/2 pathway not only regulates Egr-1 expression but also increases Egr-1 DNA binding activity (27).

The MAPKs and the PI-3 kinase/AKT pathways have emerged recently as major signaling pathways through which Tie-2 receptors regulate EC functions such as survival, migration, adhesion, proliferation and differentiation (7). In a recent study, we reported that the Ang-1/Tie-2 receptor pathway stimulates the production in ECs of the chemokine IL-8 and that this response is mediated through the PI-3 kinase and the Erk1/2 and SAPK/JNK members of the MAPKs while the p38 pathway inhibits IL-8 production through selective inhibition of Erk1/2 phosphorylation (24). Our current results indicate activated Tie-2 receptors utilize the Erk1/2 and PI-3 kinase pathways to upregulate Egr-1 expression in a similar fashion to that observed regarding IL-8 induction. We also confirm here that the p38 MAPK pathway exerts a negative influence on Egr-1

transcription and that this effect is mediated through a negative influence on Erk1/2 pathway activation. This conclusion was verified by observing a significant increase in basal and Ang-1-induced Erk1/2 phosphorylation when the p38 MAPK pathway was selectively inhibited (Figure 4.1). The inhibitory effect of the p38 MAPKs on Erk1/2 pathway has been reported in other cell types where selective activation of the p38 MAPKs causes rapid dephosphorylation of MEK1/2 (upstream regulators of Erk1/2 phosphorylation) and it has been attributed mainly to the inhibitory effect of the PP2A phosphatase on MEK1/2 phosphorylation (28). Our previous (11;19;24) and current results suggest that the Erk1/2 pathway contribute positively to Ang-1-induced survival, migration and proliferation of ECs whereas the p38 MAPKs provide an inhibitory biological switch through which other stimuli regulate the degree to which the Ang-1/Tie-2 axis modulates the above-described processes.

In addition to the Erk1/2 and PI-3 kinase pathways, we report here that Ang-1-induced Egr-1 expression was significantly attenuated by rapamycin suggesting the involvement of the mTOR network (Figure 4.1). mTOR is a serine/threonine protein kinase that regulates cell proliferation, hypertrophy, ribosome biogenesis, glucose and fat metabolism, autophagy, transcription and cytoskeleton organization (29). We have recently reported that mTOR and its downstream effector, p70S6 kinase, are activated downstream from Tie-2 receptors in HUVECs and that mTOR participates in Ang-1-induced alteration in HUVEC transcriptome (19). Very little is known about the interaction between Egr-1 and the mTOR network. During skeletal myoblast differentiation, induction of Egr-1 expression is mediated in part through the mTOR/P70S6 kinase pathway (30). The fact that the PI-3 kinase pathway could also activate the mTOR network suggests that reduction of Egr-1 expression by wortmannin in the current study could also be attributed to inhibition of the mTOR network.

It should be emphasized that Egr-1 induction by the Ang-1/Tie-2 axis was transient (Figure 4.1). We speculate that the transient nature of Egr-1 induction could be due to the binding and downregulation of Egr-1 promoter by Egr-1 itself (31). We also propose that Ang-1-induced Nab2 expression following the initial induction of Egr-1 expression could be mediated by Egr-1 itself since Egr-1 has been shown to induce the activation of the Nab2 promoter (32).

Another important finding in our study is that VEGF induces upregulation of Egr-1 expression and that a combination of Ang-1 and VEGF has an additive effect on Egr-1 expression. Although several reports have confirmed that pro-angiogenesis growth factors such as VEGF regulate Egr-1 expression, little information is available about the interactions between various growth factors in terms of Egr-1 regulation. Gerritsen *et al.* (33) have reported that both HGF and VEGF induce Egr-1 expression in ECs but the induction was less than additive when these two growth factors were combined. By comparison, the additive effects of Ang-1 and VEGF regarding Egr-1 expression suggest that both growth factors activate similar signalling pathways. Indeed, many genes, which were upregulated after 4h of VEGF treatment of HUVECs, were also upregulated by Ang-1 (19;33).

Regulation of EC migration and proliferation by Egr-1

The current study reveals that Ang-1 stimulates EC migration and that knocking down Egr-1 expression using siRNA oligos or DNAzymes attenuates Ang-1-induced EC migration (Figure 4.3). Exposure of ECs to exogenous Ang-1 protein elicits a mild increase in cell proliferation (34). To enhance the pro-proliferative effects of Angiopoietin-1 and to reproduce the *in vivo* vascular environment whereby ECs are exposed to sustained Ang-1 production derived from vascular smooth muscles, we generated HUVEC line which stably produce Ang-1 (MSCV-Ang-1) using retroviruses as described in our recent study (24). In these cells, Ang-1 induces significant cell proliferation which was associated with upregulation of Egr-1 expression (Figure 4.4). The mechanisms through which Egr-1 modulates EC proliferation and migration are still under investigation. One possibility is that Egr-1 upregulates the production of pro-angiogenesis growth factors and that these factors act in an autocrine fashion to stimulate EC proliferation and migration. This proposal is based on many reports documenting Egr-1-mediated induction of several pro-angiogenesis growth factors such as FGF-2, PDGF-A, PDGF-B, granulocyte-colony stimulating factor (G-CSF) and IGF-II in various models of *in vitro* and *in vivo* angiogenesis (14;16;35;36). The functional importance of Egr-1 in cellular proliferation induced by these factors particularly FGF-2- and G-CSF-induced cellular proliferation was confirmed using selective Egr-1 DNAzymes and

siRNA oligos (16;35). Our study confirms that Egr-1 regulates the expression of selective pro-angiogenesis growth factors such as PDGF-B and FGF-2 but is not involved in regulating others such as VEGF-A, VEGF-C and HGF (Table 4.1). This observation raises the possibility that Egr-1 contributes to Ang-1-induced proliferation and migration in these cells through the release of growth factors such as PDGF-B and FGF-2.

Another likely mechanism through which Egr-1 promotes Ang-1-induced proliferation and migration of HUVECs is the production of the chemokine IL-8 (Table 1). IL-8 is endogenously produced by ECs and it could directly promote EC proliferation and migration through activation of CXCR1 and CXCR2 (37). We have recently reported that Ang-1 elicits significant upregulation of IL-8 production in HUVECs and that this chemokine contributes significantly to Ang-1-induced proliferation and migration of these cells (24). Our current study suggests that Egr-1 is involved in the regulation of IL-8 production in ECs. Little is known about the link between Egr-1 and IL-8 in these cells. Several studies have reported that attenuation of Egr-1 expression triggers a significant reduction of IL-8 expression in the monocytic THP-1 cell line and lung fibroblasts stimulated with amyloid peptides and cigarette smoke water extract, respectively (38;39). Chromatin immunoprecipitation analysis also revealed the presence of Egr-1 binding sites in the IL-8 promoter (40). Additional studies are clearly needed to investigate the exact downstream pathways through which Egr-1 regulates Ang-1-induced proliferation and migration of ECs.

In summary, we report that Ang-1 induces the expression and activation of the transcription factor Egr-1 and that this effect is mediated through the Erk1/2, PI-3 kinase and mTOR pathways. Our results also indicate that Egr-1 contributes significantly to Ang-1-induced migration and proliferation of ECs.

4.6. ACKNOWLEDGMENTS

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Figure 4.1: Ang-1 induces the expression of Egr-1 and Nab2. Roles of the Erk1/2, p38, SAPK/JNK, PI3-kinase and mTOR pathways.

A & B: Time course of Egr-1 mRNA expression (measured with real-time PCR) triggered by COMP-Ang-1, Ang-2 and Ang-4 (each at 300 ng/ml). *P<0.05 compared with cells exposed to PBS (control).

C: Egr-1 mRNA expression after 1h of stimulation with either 300 ng/ml of Ang1 or 80 ng/ml of VEGF or combination of both. Symbols are as above.

D: Effects of pre-incubation for 1h with various inhibitors on Ang-1-induced Egr-1 mRNA levels. * P<0.05 compared with cell stimulated with COMP-Ang-1 (300 ng/ml) alone.

E: Effect of p38 inhibition on Ang-1-induced Erk1/2 activation. Cells were pretreated for 1h with PD169316 or SB203580 and were then stimulated for 15 min with COMP-Ang-1 (300 ng/ml). Cell lysates were then immunoblotted with total and phosphorylated Erk1/2 antibodies.

F: Time course of Nab2 mRNA expression in response to 300 ng/ml of COMP-Ang-1. * p<0.05 compared with cells exposed to PBS (control). N=6 measurements for each group in all panels.

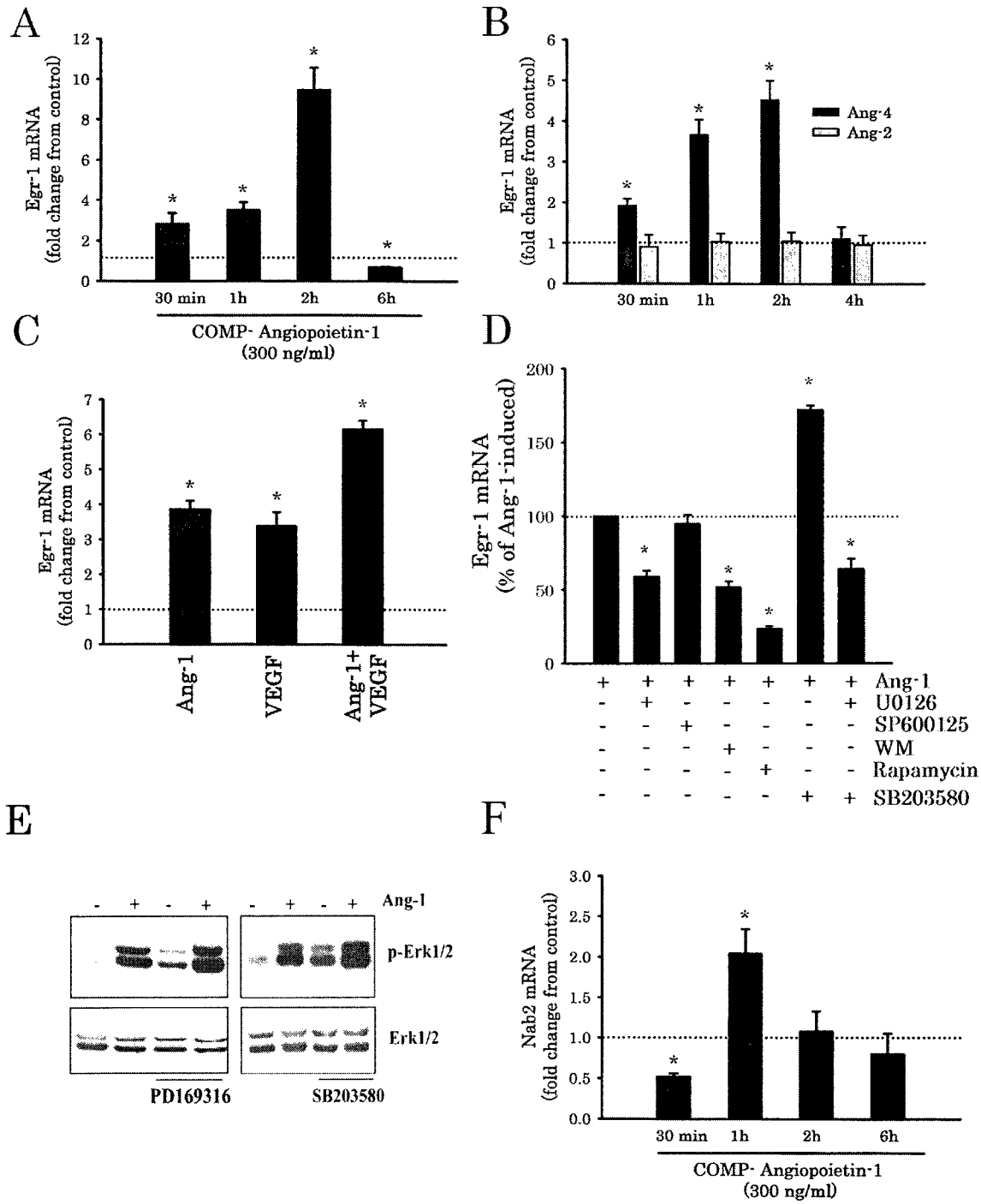
A
Egr-1 mRNA
(fold change from control)
30 min
1h
2h
6h
COMP- Angiopoietin-1
(300 ng/ml)
B
Egr-1 mRNA
(fold change from control)
Ang-4
Ang-2
30 min
1h
2h
4h
C
Egr-1 mRNA
(fold change from control)
Ang-1
VEGF
Ang-1+
VEGF
D
Egr-1 mRNA
(% of Ang-1-induced)
Ang-1
U0126
SP600125
WM
Rapamycin
SB203580
E
Ang-1
p-Erk1/2
Erk1/2
PD169316
SB203580
F
Nab2 mRNA
(fold change from control)
30 min
1h
2h
6h
COMP- Angiopoietin-1
(300 ng/ml)

Figure 4.1

Figure 4.2: Ang-1 induces the expression, nuclear accumulation and DNA binding of Egr-1 protein.

A: A representative immunoblot of Egr-1 protein in total HUVEC lysates measured after 1, 3 and 6h exposure to PBS (control) or COMP-Ang-1 (300 ng/ml).

B: Expression of Egr-1 protein in the cytosolic (C) and nuclear (N) fractions of HUVEC lysates treated for 1h with PBS or COMP-Ang-1.

C: Binding of nuclear extracts from control and COMP-Ang-1-treated HUVECs for the indicated times to specific Egr-1 DNA oligonucleotides in the absence (-) and presence (+) of anti-Egr-1 antibody. Competition with cold wild-type and mutated oligonucleotides was performed as specificity control. SS: Supershifting.

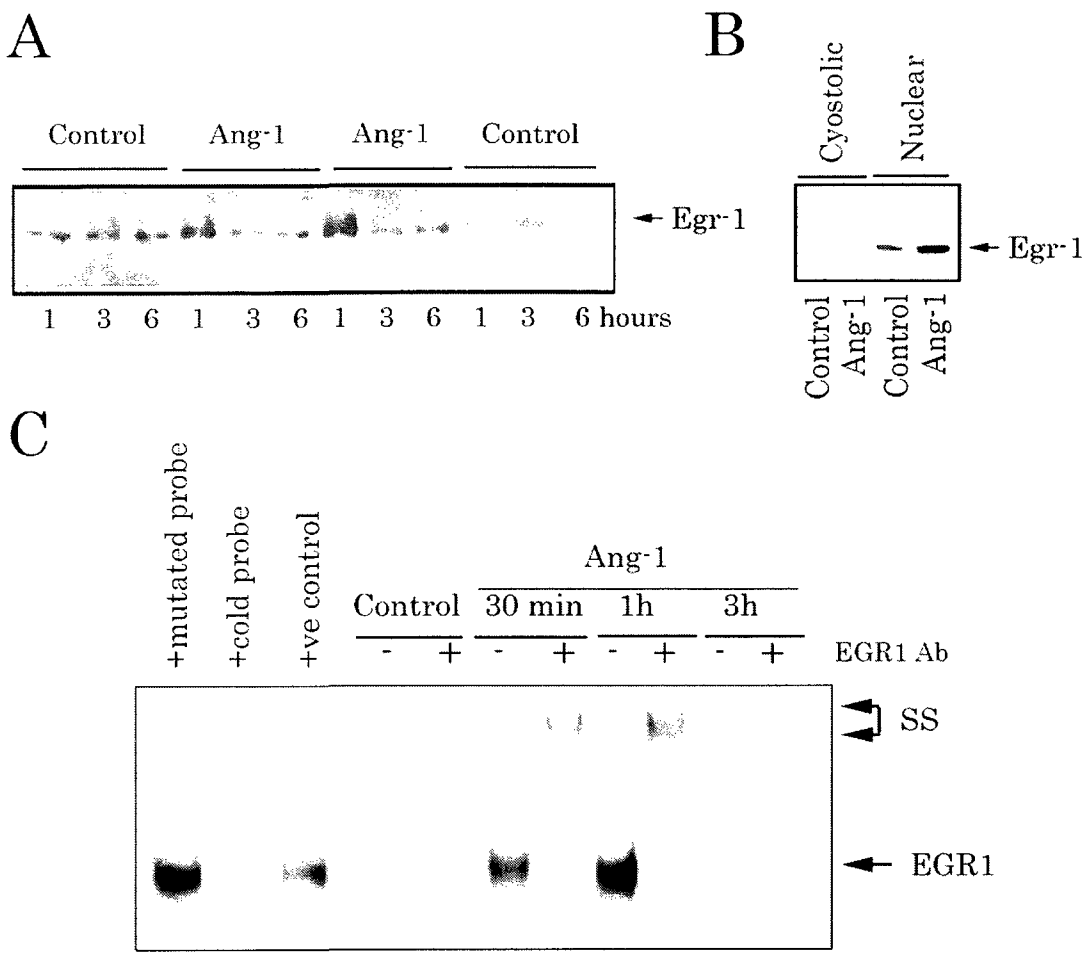
A
Control
Ang-1
Ang-1
Control
Egr-1
1 3 6 1 3 6 1 3 6 1 3 6 hours
B
Cytostolic
Nuclear
Egr-1
Control
Ang-1
Control
Ang-1
C
+mutated probe
+cold probe
+ve control
Ang-1
Control
30 min
1h
3h
- + - + - + - +
EGR1 Ab
SS
EGR1

Figure 4.2

Figure 4.3: Ang-1-induced Egr-1 plays a significant role in EC migration

A: Egr-1 mRNA levels (measured with real-time PCR) in HUVECs transfected with Egr-1 siRNA oligos or Egr-1 DNAzymes (DzF) and are expressed as percent of values measured in cells transfected with scrambled siRNA oligos and DNAzymes. *P<0.05 compared with cells transfected with scrambled siRNA or scrambled DNAzymes. N=3 in each group.

B: Wound healing in HUVECs transfected with either scrambled (DzFSCR) or Egr-1-specific (DzF) DNAzymes and treated with either vehicle (PBS) or COMP-Ang-1 for 12h. *P<0.05 compared with cells transfected with scrambled DNAzymes and exposed to vehicle. # P<0.05 compared with cells transfected with scrambled DNAzymes and exposed to COMP-Ang-1. n=6 for each group.

C: The number of HUVECs transfected with either scrambled or Egr-1-specific siRNA oligos migrating towards either PBS (vehicle) or COMP-Ang-1 placed in the lower compartment of the migration apparatus (n=6 measurements for each group). *P<0.05 compared with cells transfected with scrambled oligo and migrating toward vehicle. # p< 0.05 compared with cells transfected with scrambled oligos and migrating toward COMP-Ang-1.

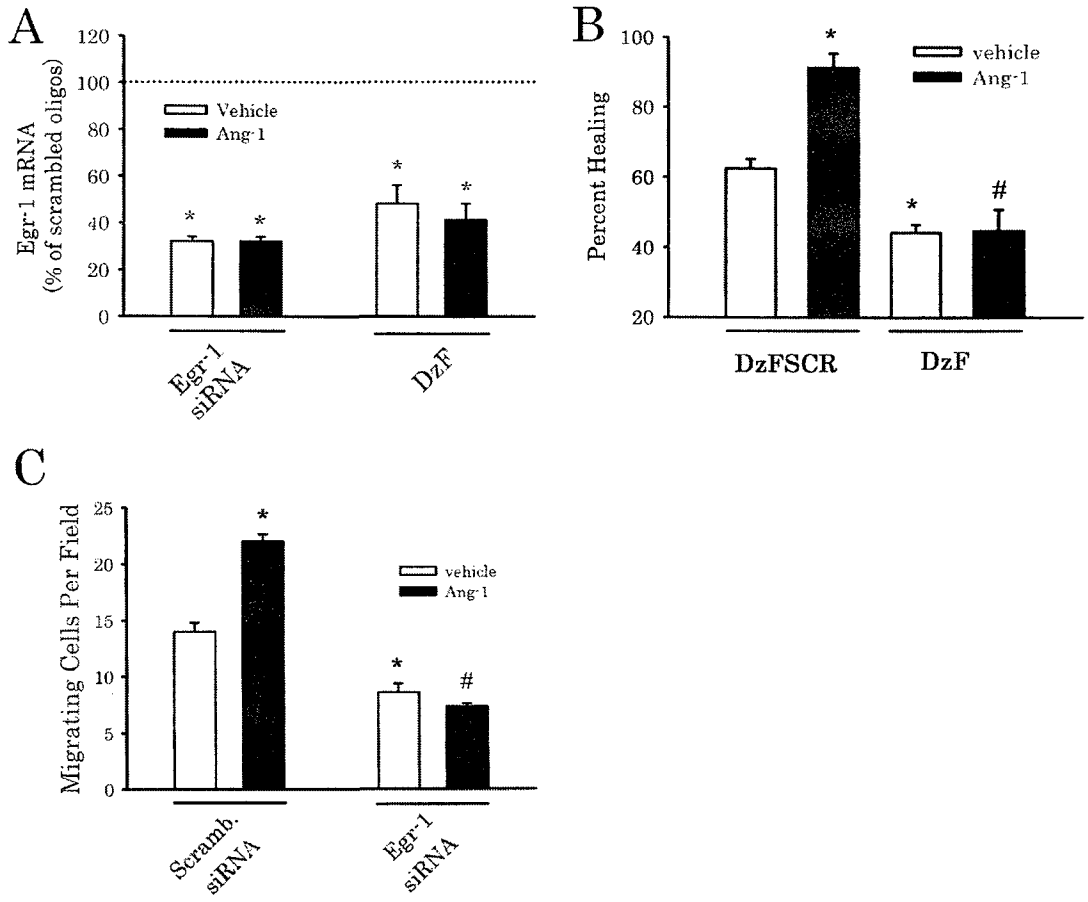
A
Egr-1 mRNA
(% of scrambled oligos)
120
100
80
60
40
20
0
Vehicle
Ang-1
*
*
*
*
Egr-1
siRNA
DzF
B
Percent Healing
100
80
60
40
20
vehicle
Ang-1
*
*
#
DzFSCR
DzF
C
Migrating Cells Per Field
25
20
15
10
5
0
vehicle
Ang-1
*
*
#
Scramb.
siRNA
Egr-1
siRNA

Figure 4.3

Figure 4.4: Ang-1-induced Egr-1 plays a significant role in EC proliferation

A: Detection of Ang-1 protein in the medium and extracellular matrix (ECM) of HUVECs stably transduced with control retroviruses (MSCV) and retroviruses expressing murine Ang-1 (MSCV-Ang-1) by immunoblotting.

B: Cell number and BrdU incorporation of MSCV and MSCV-Ang-1 HUVECs cultured for 2 days in medium containing 2% FBS containing culture medium. * $P< 0.05$ compared with MSCV cells.

C: Egr-1 mRNA expression in MSCV-Ang-1 HUVECs cultured in medium containing 15 and 0%FBS and expressed as percent of values measured in MSCV HUVECs. * $p<0.05$ compared with MSCV cells. N=6 for reach group.

D: BrdU incorporation in MSCV and MSCV-Ang-1 HUVECs transfected with either scrambled or Egr-1-specific siRNA oligos and maintained for 2 days in medium containing 2%FBS. *$P<0.05$ compared with MSCV cells transfected with scrambled siRNA oligos. #$P<0.05$ compared with MSCV-Ang-1 HUVECs transfected with scrambled siRNA oligos. N=6 in each group.

E: Cell number of MSCV and MSCV-Ang-1 HUVECs transfected with either scrambled or Egr-1-specific DNAzymes and maintained for 2 days in medium containing 2%FBS. *$P<0.05$ compared with MSCV cells transfected with scrambled DNAzymes. #$P<0.05$ compared with MSCV-Ang-1 HUVECs transfected with scrambled siRNA oligos. N=6 in each group.

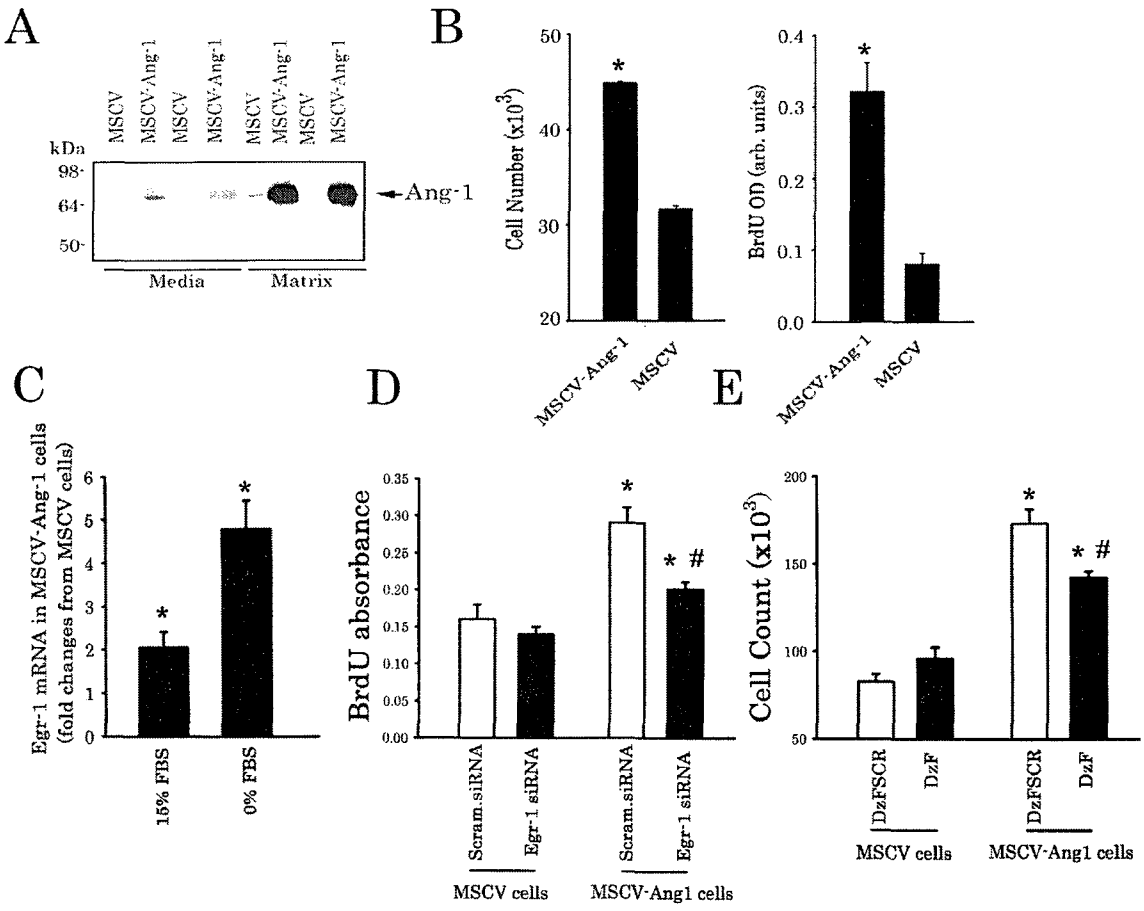
A
MSCV
MSCV-Ang-1
MSCV
MSCV-Ang-1
MSCV
MSCV-Ang-1
MSCV
MSCV-Ang-1
kDa
98-
64-
50-
Ang-1
Media
Matrix
B
Cell Number (x10^3)
50
40
30
20
*
MSCV-Ang-1
MSCV
BrdU OD (arb. units)
0.4
0.3
0.2
0.1
0.0
*
MSCV-Ang-1
MSCV
C
Egr-1 mRNA in MSCV-Ang-1 cells
(fold changes from MSCV cells)
6
5
4
3
2
1
0
*
*
15% FBS
0% FBS
D
BrdU absorbance
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
* #
Scram.siRNA
Egr-1 siRNA
Scram.siRNA
Egr-1 siRNA
MSCV cells
MSCV-Angl cells
E
Cell Count (x10^3)
200
150
100
50
*
* #
DzFSCR
DzF
DzFSCR
DzF
MSCV cells
MSCV-Angl cells

Figure 4.4

Table 4.1: Real-time PCR primers and fold changes in mRNA expression of various genes in HUVECs transfected with Egr-1 siRNA oligonucleotides.

Values are expressed as fold changes from values measured in HUVECs transfected with scrambled siRNA oligos. Cells were transfected with scrambled and Egr-1 siRNA oligos and were then exposed for 1h to basal culture medium containing 15%FBS. Values are means ±SEM. N=4 in for each measurement.

Gene change	Primers sequence (5'----3')	Product size (bp)	Fold
CXCR4	Forward 5'-AGCATGACGGACAAGTACAGG-3' Reverse 5'-GATGAAGTCGGGAATAGTCAGC-3'	309	0.80±0.12
VEGF-C	Forward 5'-TGTACAAGTGTCAGCTAAGG-3' Reverse 5'-CCACATCTATACACACCTCC-3'	183	1.10±0.15
VEGF-A	Forward 5'- CTACCTCCACCATGCCAAGT-3' Reverse 5'- CACACAGGATGGCTTGAAGA-3'	187	0.95±0.07
HB-EGF	Forward 5'- GAGAGGGCTAGCTGCTGGAA-3' Reverse 5'- TGCTTGTGGCTTGGAGGATA-3'	157	1.05±0.02
HGF	Forward 5'- GGACGCAGCTACAAGGGAAC-3' Reverse 5'- CCTTCTTCCCCTCGAGGATT	158	0.98±0.19
PDGF-B	Forward 5'- CTCGTCCGTCTGTCTCGATG-3' Reverse 5'- GGAAGAAGATGGCGATGGAG-3'	167	0.42±0.06
IL-8	Forward 5'- CTCTTGGCAGCCTTCCTGAT-3' Reverse 5'- ACAACCCTCTGCACCCAGTT-3'	242	0.63±0.11
Flt-1	Forward 5'-CACTGGGCAGCAGACAAATC-3' Reverse 5'- TCACACCTTGCTTCGGAATG-3'	109	0.34±0.04
FGF-2	Forward 5'- GAGACACCCATCCGTGAACC-3' Reverse 5'- GGCAGCGTGGTGATGCTC-3'	285	0.52±0.08
GAPDH	Forward 5'-AAGAAGGTGGTGAAGCAGGCG-3' Reverse 5'-ACCAGGAAATGAGCTTGACAA-3'	166	

CHAPTER 5

CHAPTER 5: General Discussion and Future Directions

5.1. Transcriptome of Angiopoietin-1-Activated Human Umbilical Vein Endothelial Cells

Ang-1 is an endothelial cell (EC) specific growth factor that is involved in EC survival, in promoting *in vivo* as well as *in vitro* angiogenesis and in inhibiting inflammatory responses. At the beginning of my doctoral research, appreciable amount of knowledge had been accumulated regarding the phosphorylation and activation of Tie-2 receptors, however, little was known concerning the signaling pathways and the downstream effectors such as transcription factors and soluble mediators that mediate the biological effects of this pathway. Hence, we first decided to make use of the oligonucleotide microarray technology in order to determine the transcriptome of Ang-1 treated human ECs. Ang-1 elicited coordinated responses designed to improve EC survival, proliferation and differentiation and to attenuate apoptosis and inflammation. Ang-1 induced the upregulation of several known positive regulators of angiogenesis including CXCR4, FLT-1 and VEGF-C. In addition, among the inhibited genes were two transcriptional repressors known as inhibitor of DNA binding 1 (ID1) and ID3 and the known inhibitor of angiogenesis thrombospondin-1.

In order to gain a better insight into the implications of Ang-1 induced changes in the EC transcriptome and to place the gene expression data in the context of biological pathways, we used the Ingenuity Pathway Analysis System, a knowledge-based analysis software that generates networks and pathways of regulated genes. The top functions linked with Ang-1-upregulated genes were cancer, cellular growth and proliferation, cell cycle and gene expression. A closer look at these networks revealed a number of highly connected nodes or centers, including IL-8 and Egr-1 genes. Both genes are known to be involved in promoting angiogenesis consistent with the role of Ang-1 in the vasculature. We decided to evaluate the importance of these two genes in the biological effects of Ang-1 in ECs. We first verified that Ang-1 elicits both enhanced production and secretion of IL-8 into the culture medium and upregulation of the expression and translocation of Egr-1 into the nucleus. Using gel shift assays, we confirmed that Egr-1 binds to its DNA consensus sequence consistent with its role as a transcription factor. These findings

formed the basis for my two subsequent projects presented in chapters 3 and 4. In addition, we reported that Ang-1 activates the mTOR network including the p70S6 kinase downstream from the PI3-kinase pathway. We further showed that these pathways orchestrate the induction of several proangiogenic genes downstream from Tie-2 receptors such as Egr-1, VEGF-C and Angiopoietin-like 4.

Although many of the Ang-1-induced genes identified in our study were also induced by other growth factors, such as VEGF, we demonstrated that Ang-1 could also induce a distinct set of genes. One of the interesting genes induced by Ang-1 is the transcription factor KLF-2. Further studies would likely reveal the importance of KLF-2 in the anti-inflammatory effects of Ang-1. In fact, previous studies established that KLF-2 is induced in healthy ECs by increased shear stress and that its overexpression in ECs reduces the expression of tissue factor, VCAM-1 and E-selectin induced by several pro-inflammatory cytokines (560-562). These findings suggest that Ang-1 could inhibit inflammation through induction of KLF-2.

5.2. Angiopoietin-1 Promotes Endothelial Cell Proliferation and Migration Through AP-1-Dependent Autocrine Production of Interleukin-8

Our first study demonstrated that IL-8 is released by HUVECs into the culture medium upon treatment with Ang-1. In addition to its role in inflammation, IL-8 also exerts direct proangiogenic effects on ECs, including enhancing EC migration and proliferation (274;294;297). We decided to study the role of IL-8 in Ang-1 induced angiogenic effects and explore the mechanisms through which Ang-1 induces IL-8 production in ECs. First, we demonstrated using northern blotting that Ang-1 induces in HUVECs a transient and dose-dependent increase in steady state IL-8 mRNA levels. In addition, we verified using ELISA that Ang-1 provokes the secretion of IL-8 into the culture medium in a dose- and time-dependent manner. These effects of Ang-1 on IL-8 mRNA induction and protein secretion in ECs is qualitatively similar to those of VEGF (271).

The role of Ang-1 in promoting EC migration has been well established whereas earlier studies reported Ang-1 only as a weak EC mitogen (319;346-348;353;357). However, using a model of ECs (MSCV-Ang-1 cells) whereby Ang-1 is continuously

expressed mimicking the *in vivo* vasculature, we demonstrated that Ang-1 can act as a mitogen for ECs enhancing their rate of proliferation and entry into the S phase of the cell cycle. Similar to our model of sustained Ang-1 delivery through its overexpression using a retroviral vector, Cho *et al.* demonstrated that administration of COMP-Ang1 using adenoviral vector induced long-lasting vessel enlargement due to EC proliferation (337). Interestingly, MSCV-Ang-1 cells secreted higher levels of IL-8 into the medium and migrated faster as compared to the control MSCV cells transduced with an empty retroviral vector. Accordingly, we used this cell model along with a monoclonal anti-IL-8 neutralizing antibody to study the functional role of Ang-1-induced IL-8 in promoting EC proliferation and migration. For the proliferation assay, we used two different techniques, cell count and BrdU incorporation assays. For assessing cell migration, we used both the *in vitro* wound healing and a modified Boyden chamber assay employing conditioned media derived from MSCV-Ang-1 and MSCV cells as chemoattractants. Our results showed that Ang-1-induced IL-8 plays an important role in Ang-1-induced EC proliferation and migration.

The second part of that study consisted in identifying the mechanisms through which Ang-1 induces IL-8 production in ECs. The activation of the PI-3 kinase and the Erk1/2, p38 and JNK/SAPK members of the MAPKs in ECs downstream from the Ang-1/Tie-2 receptor pathway are well characterized (341-344;409). We used pharmacological inhibitors selective for each of these pathways and determined that the PI-3 kinase, Erk1/2 and SAPK/JNK pathways are involved both in the transcriptional activation of IL-8 gene and in protein release. The involvement of these three pathways in inducing IL-8 gene transcription was further corroborated by using their selective pharmacological inhibitors in assessing the activity of a relatively short fragment of the wild type human IL-8 promoter (-133/+44) in response to Ang-1 treatment. By comparison, the p38 MAPK pathway exerted an inhibitory effect on IL-8 induction through inhibition of the Erk1/2 activation. Similarly, our group previously reported that Ang-1 enhances the survival of ECs following serum and growth factors withdrawal by simultaneously activating both the anti- (Erk1/2) and the pro- (p38) apoptotic members of the MAPKs. The strong antiapoptotic effect of the Erk pathway masks the proapoptotic effect of the p38 with a net reduction of apoptosis by Ang-1.

In terms of transcription factor activation involved in IL-8 induction, we report that AP-1 is involved in the transcriptional activation of IL-8 by Ang-1 while NFκB played no apparent role in that effect. Although many studies reported that NFκB is the predominant *trans*-acting factor responsible for IL-8 gene expression, activated Tie-2 receptors not only do not activate NFκB but also they have been shown to recruit the A20 binding inhibitor of nuclear factor-kappa beta (NFκB) activation-2 (ABIN-2) (411;412). By comparison, VEGF, which operates through an EC selective RTK and leads to an angiogenic response similar to that of Ang-1, induces IL-8 production via activation of NFκB (271). Ang-1 induces phosphorylation of c-Jun on both Ser^{63} and Ser^{73} via activation of both the SAPK/JNK and the Erk1/2 pathways. At this stage, it is unclear how the PI3-kinase pathway enhances IL-8 production since inhibition of the PI-3 kinase pathway did not elicit any effect on c-Jun activation while it suppressed the activation of the IL-8 promoter. The choice of the relatively short fragment of IL-8 promoter (-133/+44), which harbours binding sites for NFκB, AP-1 and NF-IL-6, was based on previous studies showing that this region is sufficient for maximal transcription in response to most proinflammatory stimuli (105;281;282). This region also contains a binding site for the transcription factor HIF-1α that drives in part the expression of IL-8 in response to hypoxia and hypoxia mimicking reagents (563;564). However, the full IL-8 promoter contains potential binding sites for additional nuclear factors including AP-2, IFN regulatory factor-1 (IRF-1) and hepatocyte nuclear factor-1 (HNF-1) as well as a glucocorticoid responsive element, a heat shock element and probably as yet unidentified binding sites (278). Therefore, it would be interesting to identify additional transcription factors that might be involved in the induction of IL-8 transcription by Ang-1.

IL-8 gene expression is regulated through transcriptional mechanisms and/or changes in mRNA stability (289;290;565). Our results showed that Ang-1-induced stabilization of the IL-8 mRNA in HUVECs is mediated in part through the activation of the Erk1/2 pathway. This pathway has also been shown to enhance mRNA stabilization of other genes such as the m4 muscarinic receptor by the nerve growth factor (566). Several RNA-binding proteins can associate with the adenine and uridine-rich elements (ARE) in the 3' untranslated region (UTR) of mRNA and either enhance or repress mRNA stability. These proteins include Hu antigen receptor (HuR), tristetraprolin (TTP),

KH-type splicing regulatory protein (KSRP) and T-cell inhibitor of apoptosis related protein (TIAR) (567). With the exception of HuR, these proteins have been associated with ARE-mediated RNA destabilization (567). HuR, TTP, KSRP and TIAR all bind to IL-8 ARE (568-570). HuR is implicated in enhancing the IL-8 transcript stabilization while TTP and KSRP are involved in IL-8 RNA destabilization (568;570-573). It would be interesting in future studies to identify the mRNA binding proteins that are involved in Ang-1 induced IL-8 mRNA stability. Another unexplored mechanism through which IL-8 expression is regulated is the role of histone deacetylases (HDACs). Histone modification plays a role in gene expression by releasing the tight DNA-histone interaction and revealing the transcription factors binding sites (574). In unstimulated cells, IL-8 promoter is repressed following deacetylation of histone proteins by HDAC-1 (575). In addition, agents that inhibit histone acetyltransferase activity reduce TNFα-induced IL-8 expression while agents that inhibit histone deacetylase activity enhance intracellular bacteria-induced expression of IL-8 (576;577). Whether Ang-1 acts via this mechanism remains to be determined.

5.3. Early Growth Response-1 Regulates Angiopoietin-1-Induced Endothelial Cell Proliferation and Migration

In our first manuscript, we demonstrated that Ang-1 induces in ECs the expression of the transcription factor Egr-1. Egr-1 is an immediate early gene that is induced by stress, injury, mitogens and cytokines. In turn, Egr-1 induces the expression of growth factors, cytokines and other transcription factors (109;114). In 2003, Fahmy *et al.* implicated Egr-1 in promoting *in vitro* and *in vivo* angiogenesis in response to FGF-2 (152). Based on accumulating evidence of the importance of Egr-1 in promoting the effects of vascular growth factors in angiogenesis, we decided to study the role of Egr-1 in Ang-1-induced angiogenesis and determine the mechanisms of this induction.

Initially, we verified that Ang-1 induces the mRNA and protein expressions of Egr-1 in HUVECs by real-time PCR and western blotting, respectively. Next, we evaluated the roles of the MAPK and PI-3 kinase pathways in Ang-1-induced Egr-1 and determined that the Erk1/2 and the PI-3 kinase and mTOR pathways induce whereas the p38 MAPK pathway inhibits Egr-1 expression by Ang-1. In addition, Ang-1 stimulated

the nuclear accumulation and DNA binding of Egr-1. One study showed that an interaction between Erk1/2 and Grb-2-associated binder-1 (Gab1) leads to Egr-1 nuclear accumulation. Whether a similar mechanism promotes Egr-1 nuclear translocation in response to Ang-1 remains to be determined. Downstream from the Erk1/2 and PI-3 kinase pathways, the transcription factors involved in Ang-1-induced Egr-1 expression remain to be determined. The Egr-1 promoter harbours several binding elements including serum response elements, AP-1-like site, ATF2, SP-1 and Egr-1 binding sites (171). In our previous study, we showed that Ang-1 induces the activation of AP-1 leading to enhanced transcription of IL-8. Hence, we speculate that AP-1 could play an important role in Ang-1-induced Egr-1 expression. Another mechanism through which Egr-1 expression is regulated is by Egr-1 itself. Egr-1 can bind to its own promoter and inhibit the transcription of its own gene (578). Whether Egr-1 induced downstream from the Ang-1/Tie-2 receptor pathway inhibits its own gene expression remains to be investigated. This could be accomplished by assessing the activity of the promoter containing a mutated Egr-1 binding site relative to that of the wild type promoter. Similar to other transcription factors, Egr-1 can associate with corepressors that can down-modulate Egr-1-dependent gene expression. One such corepressor is the Nab2 protein. Egr-1 has been shown to induce Nab2 expression (151). In agreement with this finding, our data showed that Ang-1 induced the expression of Nab2 with a slightly slower kinetics as compared to the induction of Egr-1.

To evaluate the functional role of Egr-1 in the biological effects of Ang-1, we used siRNAs and DNAzymes to knock down Egr-1 expression in HUVECs. Our results showed that Ang-1-induced Egr-1 plays a role in Ang-1-induced EC proliferation and migration. The mechanisms through which Egr-1 mediates Ang-1 effects are still unclear. In our previous study, we reported that Ang-1 promotes EC proliferation and migration in part through the release of IL-8. Our current study suggests that Egr-1 is implicated in the regulation of IL-8 expression in ECs. Egr-1 induction has been linked to IL-8 production in lung fibroblasts and in a monocytic cell line and Egr-1 binding sites have been identified in the IL-8 promoter. Whether IL-8 is a downstream target of Egr-1 in Ang-1-treated ECs remains to be determined. Another likely mechanism through which Egr-1 enhances Ang-1-induced proliferation and migration is the production of several growth

factors including FGF-2, PDGF-A and PDGF-B (114;152;579). In our study, we demonstrated that Egr-1 mediates the expression of PDGF-B and FGF-2. It is possible that Egr-1 participates in Ang-1-induced angiogenic effects through the release of these growth factors. However, future experiments should identify all Egr-1 target genes in Ang-1-stimulated ECs to help us further understand the Egr-1-dependent mechanisms behind Ang-1-induced angiogenesis. For that end, one would use the ChIP-on-chip technique whereby one immunoprecipitates DNA sequences in Ang-1-treated HUVECs by using antibodies specific to Egr-1 then perform DNA microarray analysis on the immunoprecipitates. Another approach would be to obtain ECs from Egr-1 knock-out mice then identify the transcriptome of these cells following activation by Ang-1. In addition, it would be interesting to show *in vivo* whether Ang-1-induced Egr-1 plays a role in Ang-1-induced angiogenesis. This could be accomplished by using Egr-1 knock-out mice in an already established *in vivo* model of vascularization such as in experimentally induced mouse hind limb ischemia. Then, we could check whether Egr-1 knock-out mice show less collateral vessels formation in response to intramuscular Ang-1 gene delivery.

Contributions to Original Knowledge

The work performed in this thesis has provided several original contributions:

Chapter 2: Transcriptome of Angiopoietin-1-Activated Human Umbilical Vein Endothelial Cells

1. The first published study profiling the gene expression elicited in cultured HUVECs in response to Angiopoietin-1 treatment.
2. Activation of mTOR in HUVECs following treatment with Angiopoietin-1.
3. Involvement of the Erk1/2, PI3-kinase and mTOR pathways in regulating the expression of pro-angiogenic genes in response to Ang-1.

Chapter 3: Angiopoietin-1 Promotes Endothelial Cell Proliferation and Migration Through AP-1-Dependent Autocrine Production of Interleukin-8

1. Identification of Interleukin-8 as a novel secreted mediator of Angiopoietin-1 effects in ECs: IL-8 partly mediates Ang-1-induced EC proliferation and migration.
2. Generation of a cultured EC model overexpressing Ang-1 that highlights the pro-proliferative effects of Ang-1.
3. Ang-1 induction of a cross-talk between the p38 and the Erk1/2 MAPK pathways whereby p38 reduces Erk1/2 activation by Ang-1.
4. Ang-1 enhancement of IL-8 gene expression through improvement of its mRNA stability.
5. AP-1 as a transcription factor activated downstream from the Ang-1/Tie-2 receptor pathway: Ang-1 phosphorylates c-Jun on both Ser^{63} and Ser^{73}.
6. AP-1, not NFκB, as the transcription factor involved in Ang-1-induced IL-8 production.

Chapter 4: Early Growth Response-1 Regulates Angiopoietin-1-Induced Endothelial Cell Proliferation and Migration

1. Egr-1 as a novel transcription factor induced and activated by Ang-1 in ECs.

2. Role of mTOR and p70S6 kinase as novel kinases activated by Ang-1 in ECs and that play a role in Ang-1-induced gene expression, more specifically Egr-1.
3. Egr-1 as a mediator of Ang-1-induced EC proliferation and migration.

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APPENDIX